TRANSFORMATION OF DISTINCT MAMMARY EPITHELIAL CELL POPULATIONS INFLUENCES BREAST CANCER PHENOTYPE

by

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ABSTRACT

The mammary gland is a branched, secretory organ that that is composed of diverse cells types. The populations that comprise the mammary epithelium include mammary stem cells, lineage-restricted progenitors, and differentiated luminal and basal cells. Breast cancer develops when one or a subset of these epithelial cells undergoes transformation, resulting in disease that can vary broadly in pathology and prognosis. For example, gene expression analysis of a large cohort of breast cancers has classified tumors into distinct subtypes, including: HER2-positive, Basal-Like, Luminal A, and Luminal B. Furthermore, hormone receptor status is also used in the clinic to classify tumors and define the course of treatment. Factors that may contribute to this broad breast cancer diversity are still unclear; however, we hypothesize that breast cancer phenotype may be influenced by intrinsic properties of the originating cell.

Therefore, to determine whether specific cells types influence breast cancer pathology, the goal of this dissertation has been to develop a series of mouse models of breast cancer that can be used to target oncogene expression to distinct mammary epithelial cell (MEC) lineages. One such model was generated by infecting mouse MECs with a lentivirus expressing the polyoma virus middle T antigen oncogene, then cells were enriched for specific cell lineages using fluorescence activated cell sorting, and were orthotopically transplanted to generate tumors. Resulting tumors were classified by histology, estrogen receptor status, molecular subtype, and metastatic propensity. Our results demonstrated that each MEC population we evaluated could give rise to diverse tumor types; however, we also determined that MEC populations exhibited differences in their propensity for tumor histology, molecular subtype, and metastatic potential.

Furthermore, we developed an additional approach of targeting specific gene expression to distinct MEC populations. In this system, lentiviral constructs were designed to express a specific gene after Cre-mediated recombination. Then, MECs isolated from mice that express Cre

through cell lineage-restricted promoters were infected with the lentivirus and orthotopically transplanted or analyzed by three-dimensional culture. We validated this approach by targeting fluorescent Tomato reporter and polyoma virus middle T antigen oncogene expression to specific MEC populations *in vitro* and *in vivo*.

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3D	Three-dimensional
AHR	Aryl hydrocarbon receptor
AKT	Protein kinase B
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
CTC	Circulating tumor cell
DMBA	7, 12-Dimethylbenz(a)anthracene
EF1α	Elongation factor-1 alpha
ELF5	E74-like factor 5
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ER+	Estrogen receptor positive
ER-	Estrogen receptor negative
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFP	Enhanced green fluorescent protein
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
H2B-EGFP	Histone H2B-fused enhanced green fluorescent protein
H&E	Hematoxylin and eosin
HBSS	Hanks Balanced Salt Solution
HER2	Human epidermal growth factor receptor 2
IDC-NOS	Invasive ductal carcinomas not otherwise specified
IGF1	Insulin-like growth factor
IHC	Immunohistochemistry
ILC	Invasive lobular carcinomas
K8	Keratin 8
K14	Keratin 14
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
mG	Membrane-targeted green fluorescent protein
MMTV	Mouse mammary tumor virus
mT	Membrane-targeted Tomato
MYC	c-Myc protooncogene
PI3K	Phosphatidylinositol-3-OH kinase
PBS	Phosphate buffered saline
PRB	Petinoblastoma protein
PYMT	Polyomavirus middle T antigen
RAS	Rat sarcoma
SM22α	Smooth muscle 22-alpha
SMMHC	Smooth muscle myosin heavy chain
SV	Simian virus
TAG	T antigen

TEB	Terminal end bud
WAP	Whey acidic protein
WK1	One week post transplantation
WK4	Four weeks post transplantation

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CHAPTER I

INTRODUCTION

Mammary Gland Development

The mammary gland is a unique mammalian organ specialized for the nourishment of newborn animals. It consists of diverse cell populations, including a densely branched epithelium that performs most of the organ's essential secretory functions, and a variety of supporting cells such as adipocytes, vascular endothelial cells, fibroblasts, and immune cells. Proper development and morphogenesis of the ductal network are critical for generating a large surface area to produce an adequate milk supply for offspring during lactation (1).

Most of what is known about human breast development has been discovered in studies analyzing mouse mammary glands. The majority of development for building the densely branched mouse mammary gland epithelium take place during two key postembryonic stages: ductal morphogenesis during puberty; and alveolar differentiation during pregnancy and lactation (1). Figure 1.1 summarizes the key stages of postnatal mouse mammary gland development, which are discussed below (adapted from Macias and Hinck, 2012 (1)).

The initial stages of mammary gland growth begin by the middle of mouse embryogenesis, when cells migrating from the ectoderm form mammary placodes along the ventrally located mammary lines. Mesoderm-derived fibroblast cells also form a layer around the epithelial cells (2,3). By embryonic day 14, the rudimentary mammary gland develops into a bud that connects to the epidermis and extends into the dermal mesenchyme (1). As the mammary epithelium continues to grow, it reaches a fat pad derived from subcutaneous mesenchymal cells and begins to branch, forming a rudimentary ductal network containing a lumen (4-6). After this formation, most of the remaining growth and development occurs at puberty (Figure 1.1).

At birth, the mammary gland continues to grow in proportion with the animal's body size until puberty. Once the mouse reaches puberty at 4 weeks, growth hormone (GH) produced by the pituitary gland induces insulin-like growth factor 1 (IGF1) secretion from the liver and mammary gland stroma (7,8). Then, IGF1, along with estrogen secreted from the ovaries (9,10), promote mammary epithelial cell proliferation at the ductal terminal end buds (TEBs). As the extending TEBs invade and fill the fat pad, the primary ducts undergo branching morphogenesis by dividing at TEBs to form secondary lateral branches (11).

After the mouse reaches adulthood at 12 weeks, it begins to go through cycles of estrus. At this time, cycling of the ovarian hormone progesterone stimulates tertiary branch growth to prepare the mammary epithelium for expansion during pregnancy (Figure 1.1). With pregnancy, progesterone and pituitary gland-secreted prolactin hormones stimulate further branching and proliferation of milk secreting alveolar cells. Then, alveolar cells fill up the entirety of the mammary fat pad for maximal milk production (12-16).

Lactation completes after the pups stop suckling, or are weaned. Weaning causes mammary gland engorgement and initiation of the first stage of involution, which is reversible within 48 hours by suckling. During this stage, alveolar cells begin to die and are shed into the ductal lumens (17-19). The second stage of involution occurs when alveolar cells collapse, a week after weaning, and a massive wave of apoptosis leads to mammary gland tissue remodeling into a pre-pregnancy state (20-22).

In sum, the major mammary developmental events take place during puberty and pregnancy. These processes are essential for the generation of a highly branched ductal network for subsequent nourishment of mammalian newborns. Furthermore, the cycles of substantial proliferation, apoptosis, and tissue remodeling during pregnancy and involution indicate that the mammary epithelium is highly proliferatory and regenerative.

Mammary Epithelial Cell Hierarchy

Much of what we know about the cellular architecture of the breast epithelium comes from analyzing mouse mammary glands. Both the human and mouse mammary epithelium consists of a branched network of bilayered ducts that are predominantly composed of luminal

and basal cells (Figure 1.2A). Luminal cells populate the inner layer of mammary ducts and border a central lumen, whereas the outer layer, adjacent to the basement membrane, is populated by basal cells. This basal layer also contains rare mammary epithelial stem cells (23). The specific functions and characteristics of individual mammary epithelial cell populations are described below.

Mammary stem cells and progenitors

The mouse mammary gland is a highly regenerative organ. This was highlighted by early transplantation experiments, which demonstrated that fragments of mammary ducts implanted into epithelium-free, or "cleared", mammary fat pads could reproduce the ductal network. These studies provided evidence for the presence of a mammary stem cell population in adult mammary tissue, a population that was later shown to be distributed throughout the length of the ductal structure (24-26).

A distinct population of mammary gland repopulating cells, or stem cells, was initially described to reside in terminal end buds of developing ducts during puberty (11). Later fluorescence activated cell sorting (FACS) experiments were able to enrich for subsets of highly regenerative basal cells from the adult mammary epithelium that expressed high levels of the cell surface markers CD49f, CD29, and low to moderate levels of CD24 (27-30). However, these studies only enriched for mammary stem cells that were intermixed with basal cells, and did not isolate a pure population of stem cells. Based on mammary transplantation and repopulating assays, Shackleton et al. estimated that one out of 18 of these enriched basal cells was actually a mammary stem cell (27).

To further delineate mammary stem cells from more differentiated basal populations, transgenic mice driving reporter expression through established stem cell promoters were also used to identify mammary stem cells. For example, the Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) promoter, a Wnt target gene, is active in stem cells of several adult tissues, including the intestinal epithelium, skin, and kidney (31). In the mammary gland, Plaks et al. also demonstrated that LGR5-positive cells have a high repopulating capacity (32). However, other studies report that both LGR5-positive and negative mammary epithelial cells have stem

cell activity, indicating that the mammary stem cell population is heterogeneous (33,34). Furthermore, stem cells isolated from fetal and adult mammary glands also have different properties. Unlike adult mammary stem cells, which express basal cell surface markers and cytokeratins, fetal mammary stem cells display both basal and luminal lineage properties, and have a higher regenerative capacity (35,36).

To add to the complexity of the mammary progenitor compartment, recent lineage tracing experiments using inducible Cre reporter mice have presented conflicting results about which MEC populations drive the propagation and maintenance of the ductal network during postnatal development. Van Keymeulen et al. demonstrated that after birth, lineage committed basal and luminal cell progenitors promote mammary gland growth and maintenance during puberty and pregnancy (37). However, recent studies have also demonstrated that a bipotent basal progenitor may be responsible for the maintenance of the mammary epithelium during postnatal development (34). Further studies need to be conducted to definitively differentiate between adult mammary stem cells, bipotent and multipotent progenitors. Figure 1.2B (adapted from Visvader and Stingl, 2014) summarizes the current hypothesized mammary epithelial cell hierarchy (38).

Basal cells

Basal cells populate the peripheral layer of the mammary duct epithelium and interact with the basement membrane. This layer consists of basal progenitors and more differentiated myoepithelial cells. The role of the myoepithelial cell population is to utilize smooth muscle contractile fibers to propel milk through the ductal network during lactation. Furthermore, the basal/myoepithelial cell layer acts as a barrier between the stroma and the mammary epithelium, which may suppress tumor growth and invasion into the basement membrane during mammary gland pathogenesis (39,40).

As described above, basal/myoepithelial cells represent the major component of the mammary epithelial cell population which expresses high levels of cell surface markers CD49f, CD29, and low to moderate levels of CD24. There are some differences in the mammary repopulating capacities of basal cells expressing variable levels of FACS-detectable cell surface

markers; otherwise, stem cells, basal progenitors, and differentiated myoepithelial cells are difficult to distinguish (27-30).

Morphologically, basal cells differ based on their location in the ductal network. Along the length of the ducts, basal cells are shaped like spindles and form a single continuous layer adjacent to the luminal cell compartment. At ductal termini, basal cells adopt a stellate shape and form web-like nets surrounding the terminal luminal cells, and alveoli during lactation (39,41).

Much of basal cell function and shape is coordinated by smooth muscle actin and myosin fibers. During lactation, basal cells undergo further morphological changes to completely surround milk-producing alveoli, and contraction of the myoepithelial smooth muscle fibers is essential for the facilitation of milk ejection (41-43). Despite resembling and acting like smooth muscle cells, the basal lineage is derived from the ectoderm and can be identified by its expression of epithelial cytokeratins 5, 14, and 17 (37,39,44).

Interactions between basal cells and their extracellular environment also play a critical role in their function. Basal cells are directly adjacent to the basement membrane, which surrounds the mammary epithelium and is composed of collagens and laminins (45). For example, integrins expressed by basal cells interact with the basement membrane to coordinate ductal branching morphogenesis and alveolar growth (46). Furthermore, basal cell-specific desmosomal junctions play a role in organizing the basal and luminal cell layers(47). The maintenance of apicobasal polarity by basal cell tight junctions may help inhibit tumor invasion into the basement membrane during breast pathogenesis (40).

Luminal cells

The luminal cell population makes up the inner layer of the mammary duct. One function of the luminal cell compartment is to maintain mammary duct structural integrity. In addition, subsets of luminal cells respond to external hormonal cues such as estrogen, progesterone, and prolactin that activate proliferation during postnatal developmental stages and promote differentiation into milk secreting alveolar cells during pregnancy (1,23,38).

The luminal compartment is composed of diverse populations of cells (Figure 1.2B). FACS analyses have demonstrated that luminal progenitors, alveolar progenitors, and hormone

receptor positive and negative populations can be distinguished based on differential expression of cell surface markers (38). For example, a majority of luminal cells express an abundance of CD24 and low levels of CD49f (27-29); however, differential expression of CD133 can be used to separate luminal cells into progenitor (CD133-) and more mature estrogen receptor positive (ER+, CD133+) populations (48). The distinct luminal cell populations vary in their survival capacity in colony forming assays, but typically do not form substantial outgrowths upon transplantation into cleared mammary fat pads (37,38).

Cell lineage tracing studies using transgenic Cre reporter mice have also been used to identify subsets of luminal cells. The promoters for E74-like factor 5 (Elf5), a transcription factor functioning downstream of prolactin signaling (49), and keratin 8 are active in luminal progenitors; however, it is not clear whether both of these promoters are active in the same populations (34,37,50). The hormonally-regulated whey acidic protein (WAP) promoter is active in luminal populations that differentiate into milk producing alveolar cells during late stages of pregnancy. Lineage tracing experiments have demonstrated that cells which expressed WAP can survive involution, and serve as a pool of alveolar precursors for subsequent rounds of pregnancy. However, WAP-positive cells do not contribute the differentiation of all luminal cell types, including the ER+ luminal population (51,52). Another hormone responsive promoter, the mouse mammary tumor virus (MMTV) long terminal repeat is active in large proportions of luminal cells (53). Also, the Notch3 promoter is active in transiently quiescent luminal progenitors that give rise to both ER+ and ER- ductal cells (54).

Overall, the luminal compartment is composed of heterogeneous populations of cells. These cells include luminal lineage-restricted progenitors, alveolar precursors, and mature hormone receptor positive and negative cells. Further lineage tracing studies need to be performed to clearly define the broad number of potential luminal progenitors and their contribution to mammary duct maintenance.

Transcriptional regulators of mammary epithelial cell fate

The proper balance of stem, progenitor, and differentiated mammary epithelial cell populations is regulated by multiple transcription factors. Stem cell maintenance relies heavily on p63, Slug, Sox9, and Twist activities (55-57). Loss of these transcription factors can promote a luminal cell fate, and their overexpression leads to an increase of cells with basal and stem cell properties (58,59). Multiple cellular signaling components can downregulate stem cell transcription factors to promote luminal cell lineage commitment. For example, the Notch pathway inhibits p63 expression (60,61). In addition, ELF5 directly blocks Slug transcription to enhance luminal and alveolar cell development in response to progesterone signaling (50,62-64). Other transcription factors involved in luminal and alveolar cell commitment include Stat5a and Gata3 (65-68). Improper activities of these cell fate regulators can inhibit mammary gland development or induce pathogenesis (69).

Breast Cancer Diversity

Breast cancer is a heterogeneous disease. This is evidenced by recent genetic profiling studies that have identified at least five distinct molecular subtypes of breast cancer (70,71). In the clinic, breast tumors are also classified by their histological grade, hormone receptor status, and growth factor receptor expression (72). Both molecular and histological characteristics of breast tumors help predict disease outcome and decide the course of treatment. The purpose of this section is to give an overview of the major breast cancer subtypes and highlight the broad heterogeneity that this disease presents.

Before the development of molecular classification techniques, tumor histology has been the gold standard for describing breast cancer subtype, and is still used today. Histologically, most breast tumors are classified into two major groups, these include invasive ductal carcinomas not otherwise specified (IDC-NOS) that make up 80% of breast cancers and develop from ductal cells, and invasive lobular carcinomas (ILC) that originate from milk producing glands (73). However, this histological classification system is very broad and there is debate over which type has a better prognosis. One study reported that patients with ILC are typically older, have well-

differentiated estrogen receptor-positive tumors with reduced vascular infiltration, and have a better prognosis than IDC-NOS patients (74). Conversely, a later study demonstrated that there is no difference between the outcomes of patients with ILC and IDC-NOS (75). However, several rare IDC-NOS histologies have some prognostic value. For example, well-differentiated cribriform, tubular, and papillary carcinomas have good prognoses and typically do not require aggressive treatment (73,76-78). However, patients with poorly differentiated metaplastic carcinomas that contain spindloid, squamous, or lipid-rich features have poorer overall survival rates (73,79).

In addition to tumor histology analyses, specific tumor cell markers are also used to characterize breast cancers. These include estrogen and progesterone hormone receptors, and their expression can identify cancers that respond to endocrine therapies. Patients are considered for endocrine therapies when at least 1% of their tumor cells stain positive for estrogen or progesterone receptors. These patients typically do well in response to hormone receptor inhibitor treatments, and further chemotherapy is administered depending on lymph node status and levels of Ki67 proliferation marker staining (80-84). In addition, human epidermal growth factor receptor 2 (HER2) status is also taken into consideration during breast cancer evaluation. Tumors positive for HER2 can be treated with HER2 receptor inhibitors (82,85,86). Sadly, there are no targeted therapies for triple negative breast cancers, which are typically hormone receptor and HER2 negative. Therefore, further studies need to be performed to develop more effective treatments for patients with triple negative tumors.

Furthermore, Perou and colleagues demonstrated that microarray gene expression analysis could also classify breast cancer into distinct and clinically relevant subtypes. Based on their hierarchical clustering methodologies, at least five distinct tumor types were identified, including Basal-like, HER2-positive, claudin low, luminal A, and luminal B (70,71,87,88).

The least aggressive of the molecular breast cancer subtypes is luminal A. The well-differentiated histology status of most luminal A tumors correlates with this cancer's typically good prognosis (78). Also, these tumors are hormone receptor positive and have luminal cell-specific gene expression patterns, which include hormone receptors and downstream targets,

transcription factors involved in luminal lineage commitment, and luminal cell specific cytokeratins (87,89). Due to their luminal-like nature, the growth of luminal A type tumors relies heavily on estrogen receptor signaling (90). In addition, these cancers frequently harbor mutations in the phosphatidylinositol-3-OH kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, which may be important for noncanonical estrogen receptor signaling (89,90).

Another hormone receptor positive breast cancer with luminal cell features is luminal B. Although luminal B tumors share some gene expression patterns and mutations with luminal A subtypes, it is a much more aggressive cancer. Some factors that make luminal B cancers more aggressive include higher expression of proliferation markers, increased rate of mutations in the p53 tumor suppressor pathway, and higher overall histologic grade (88,89,91,92). Furthermore, these tumors do not respond well to endocrine therapies (93,94); however, potential inhibitors targeting components of the PI3K and epidermal growth factor receptor (EGFR) pathways are currently under investigation (95,96).

Amplification of the tyrosine kinase receptor HER2 drives the growth of HER2-positive subtype tumors. HER2 signals by forming dimers with other EGFR family receptor tyrosine kinases, and activates downstream MAPK and PI3K pathways (97,98). As a result, much of the gene expression profile characterizing this molecular breast cancer class consists of downstream signaling components of the HER2 pathway (87,89). Furthermore, more recent gene expression analyses have also demonstrated that there may be two subclasses of HER2-positive cancers, one being estrogen receptor positive with luminal characteristics and the other having more basal features (89). HER2-specific inhibitors are available in the clinic (99-103); however, most patients with advanced disease relapse after treatment due to mutations in HER2, increased signaling through analogous receptor tyrosine kinase pathways, or loss of cell cycle control. Combination therapies targeting both HER2 and other parallel signaling components are currently under investigation (104).

Finally, triple negative breast cancers, which express the lowest levels of hormone receptors and HER2, have been classified into two molecular types. These include highly aggressive but closely related Basal-like and claudin low tumor classes (70,87). Overall, the

tumor morphology for these cancers is typically very metaplastic, with claudin low tumors frequently displaying a mesenchymal-like spindloid histology (70,78).

The Basal-like breast cancer group is more specifically characterized by having similar gene expression and cytokeratin profiles as normal basal/myoepithelial cells (87). In addition, the majority of Basal-like tumors have p53 mutations, high PI3K pathway activation, and increased cell proliferation signatures (89). The Basal-like cancer group also includes tumors with inactivating mutations in the tumor suppressor breast cancer genes 1 and 2 (BRCA1 and BRCA2), which are important components of DNA damage repair complexes (89,105).

The second triple negative breast cancer subtype consists of claudin low tumors that have a decreased expression of tight junction claudin and E-cadherin proteins. Furthermore, claudin low tumors upregulate genes involved in epithelial-to-mesenchymal transition and stem cell maintenance (50,70). Unfortunately, there are no targeted therapies for these highly aggressive Basal-like and claudin low breast cancers; therefore, further research needs to be performed to identify early detection markers and more specific treatments for these tumor types.

The Tumor Cell of Origin Hypothesis

Many cancers can consist of several distinct tumor subtypes that vary in clinical outcome. This intertumor heterogeneity can be explained by two different hypotheses: the mutation model proposes that accumulation of various genetic alterations define the tumor phenotype regardless of the initially transformed cell type (Figure 1.3A, adapted from Visvader, 2011 (106)), and the tumor cell of origin hypothesis proposes that the intrinsic properties or differentiation potential of the initially transformed cell population influence the resulting tumor phenotype (Figure 1.3B, adapted from Visvader, 2011 (106)). In addition, these two different hypotheses may not be mutually exclusive.

Although both the genetic mutation and tumor cell of origin hypotheses may influence tumor heterogeneity, multiple lines of evidence suggest that specific populations of a tissue's cellular hierarchy contribute to several distinct hematopoietic and solid cancer types. For example, hematopoietic stem cells harboring BCR-ABL mutations are the cells of origin of chronic myeloid leukemia (107,108). Also, more differentiated hematopoietic T-cell progenitors give rise

to T-cell acute lymphoblastic leukemia (109). Tumor cells of origin have also been identified in several solid tissue cancers. For instance, organ-specific stem cells give rise to intestinal and stomach cancers (110-113). However, various epidermal cell types contribute to distinct skin cancers: basal cell carcinomas can arise from interfollicular, infundibulum, and bulge cells; but squamous cell carcinomas originate specifically from bulge cells (reviewed in (114)). Furthermore, several studies suggest that different types of breast cancer may have specific cells of origin.

Comparative gene expression analyses have demonstrated that there is a close relationship between distinct breast cancer subtypes and specific mammary epithelial cell populations. Basal and stem cells resemble the genetic profiles of claudin low tumors, whereas luminal progenitors are most closely related to Basal-like breast cancers. Furthermore, luminal A type tumors rely on the estrogen receptor signaling pathway for proliferation, and express high levels of differentiated luminal cell markers (70,87,88,115). These findings are correlative, but inherent tumor and cellular properties suggest that different breast cancers develop from specific populations of cells.

Experimental evidence further supports the tumor cell of origin hypothesis in breast cancer. Ince et al., generated unique tumor types from two distinct mammary epithelial cell populations, demonstrating the transformed cell type influences cancer phenotype (116). In addition, the luminal progenitor population is proposed to be the tumor cell of origin of BRCA1-mutant Basal-like breast cancers. This was initially shown by demonstrating that luminal, but not basal or stem cells, isolated from pre-neoplastic lesions of BRCA1 mutation carriers were predisposed to transformation (115). Then, later transgenic mouse mammary tumor models demonstrated that BRCA1-mutant luminal progenitors give rise to Basal-like tumors, whereas basal cells carrying the same mutation produce myoepitheliomas, which are rare in breast cancer (117). Further analyses need to be performed to determine if other breast cancer subtypes have a specific tumor cell of origin. Alternatively, it may be found that strong oncogenic drivers give rise to specific breast cancers regardless of the initially transformed cell population.

Mouse Models of Breast Cancer

As discussed above, breast cancer is not a single disease, but consists of molecularly and pathologically diverse subtypes that require distinct treatment approaches. Experimental models that can recapitulate this heterogeneity are necessary in order to investigate the mechanisms driving disease progression. Such studies may lead to the discovery of more effective therapies for each breast cancer type. Over the past several decades, a variety of transgenic, mutagen-activated, and transplantation mouse mammary tumor models have been generated to achieve this goal. The purpose of this section is to provide an overview of several current mouse mammary tumor models, and to highlight their advantages and limitations in replicating breast cancer pathology.

MMTV transgenic mouse models

The mouse mammary tumor virus (MMTV) is a murine retrovirus transmitted through lactation. MMTV can activate spontaneous mammary tumor growth in mice by disrupting host tumor suppressors or upregulating proto-oncogenes after insertional mutagenesis (118-121). MMTV promotes tumor growth predominantly in the mammary epithelium, due to the hormone responsive element in the long terminal repeat (LTR) of the MMTV promoter (122). Because of this high tissue specificity, several transgenic models have been developed using the MMTV promoter to drive oncogene expression in the mouse mammary gland (123-125).

One such oncogene is the polyoma virus middle T antigen (PYMT), and its expression through the MMTV promoter results in rapid mammary tumor growth in 100% of MMTV-PYMT transgenic mice (125,126). PYMT drives tumorigenesis by acting as a membrane-anchored scaffold for several cellular kinases and GTPases. This interaction leads to activation of Rat sarcoma (RAS) and protein kinase B (AKT) signaling pathways, followed by rapid cellular transformation (Figure 1.4, adapted from Rodriguez-Viciana et al., 2006 (127)) (128). The advantage of the PYMT oncogene is that it activates similar pathways that are misregulated or mutated in most breast cancers (89). Furthermore, progression of MMTV-PYMT tumor growth is similar to breast cancer. MMTV-PYMT tumor development begins with hyperplastic mammary epithelial lesions that invade into the ductal lumens and fat pad tissue. As tumors progress, they

gradually lose cellular organization and hormone receptor expression. At later stages, metastases occur in the lungs (129). Although MMTV-PYMT mice give rise to very predictable tumor pathologies, this model does not fully recapitulate the disease heterogeneity observed in breast cancer. A majority of MMTV-PYMT tumors consist of solid adenocarcinoma histology with luminal gene expression profiles (71,129).

To model HER2-positive breast cancer, the MMTV promoter has also been used to express the activated or wildtype rat homolog of HER2 (Neu) in the mouse mammary gland (98,130,131). Interestingly, when wildtype Neu is expressed, it frequently acquires activating mutations, resulting in more potent tumor growth (132-134). Similarly, HER2 amplifications and mutations that lead to receptor dimerization and activation are also observed in the human disease (134-138). These studies have helped to confirm that HER2 dimerization and activation drives tumor growth in HER2-positive breast cancers. Furthermore, just like the human counterpart, MMTV-Neu tumors are highly aggressive and frequently metastasize to the lungs (125). One drawback of this model is that unlike in breast cancer, MMTV-Neu tumors are very homogenous and molecularly luminal (71). MMTV promoter activity in subsets of luminal cell populations may contribute to this luminal tumor characteristic (53); however, the tumor cell of origin for HER2 breast cancer is yet to be defined.

The MMTV promoter has also been used to express Wnt1 (139). Wnt1 is a key component of the Wnt pathway that is involved in stem cell maintenance and proliferation through the activation of the β-catenin transcription factor (140,141). Tumors develop in about 50% of MMTV-Wnt transgenic mice within six months post birth, and typically display differentiated acinar histologies (139,142,143). Also, metastases frequently develop in lymph nodes and lungs after primary tumor excision, indicating that metastatic seeding occurs early during primary tumor growth (139). Unlike the other MMTV mammary tumor models discussed above, MMTV-Wnt tumors are molecularly basal (71). These findings suggest that activation of Wnt signaling may drive basal breast cancer development, possibly due to expression of genes involved in stem cell maintenance. Interestingly, increased nuclear localization and activity of β-catenin is detected in

high percentages of breast cancers, and is associated with aggressive disease and poor clinical outcomes (144-147).

Despite the vast application of the MMTV promoter in generating transgenic mouse mammary tumor models, some limitations of these models should be emphasized. Although, MMTV is primarily active in the mammary epithelium, MMTV can also drive gene expression in lungs, kidneys, salivary glands, seminal vesicles, T cells, testes, and prostate (148,149). Therefore, care needs to be taken in examining metastases at secondary tumor growth sites. Furthermore, as stated above, MMTV is active in subsets of hormone-responsive luminal cells and not all mammary epithelial cell types (53); as a result, MMTV driven tumors may not represent the vast tumor heterogeneity observed in breast cancer. It should be noted, however, that expression of different oncogenes using the MMTV promoter does promote the growth of distinct tumor types. These findings indicate that the oncogene plays a role in influencing tumor subtype.

WAP transgenic mouse models

The whey acidic protein (WAP) promoter is regulated by lactogenic hormones and is primarily active in the mammary luminal epithelial cells during mid-pregnancy (51,150). Similar to MMTV, the mammary epithelial tissue specificity of this promoter has made it a useful tool in generating tissue-specific transgenic mouse mammary tumor models. Several oncogenes have been expressed using the WAP promoter to assess their ability to drive mammary tumorigenesis.

One of these is the c-myc protooncogene (MYC). MYC is a multifunctional transcription factor that is required during development and regulates many cellular processes, including apoptosis, proliferation, and metabolism (151,152). Mutations and amplifications of MYC have been reported in multiple malignancies, including breast cancer (153-157). In addition, recent studies show that luminal B and Basal-like breast cancers have the highest rates of MYC amplification and pathway activity (89). MYC expression in the mouse mammary gland under the control of the WAP promoter results in mammary tumors after two rounds of pregnancy in 80% of mice (158). However, unlike Basal-like breast cancer, WAP-MYC tumors typically consist of solid adenocarcinomas histology with a luminal molecular profile (71,158).

The WAP promoter has also been used to express oncogenes driving tumor growth through similar mechanisms that are misregulated in breast cancer. These include int3, which is a truncated form of Notch4, and the Simian Virus (SV) 40 T antigen (TAg) (159,160). Int3 was initially identified as a frequent integration site for the murine retrovirus MMTV that led to spontaneous tumor growth (161); and TAg transforms cells by inhibiting p53 and retinoblastoma protein (pRB) tumor suppressor pathways (162,163). While WAP-Int3 tumors predominantly display solid adenocarcinoma histology, WAP-TAg mice give rise to histologically diverse tumor types (159,164). In addition, both of these models give rise to molecularly luminal tumors (71).

In sum, the hormone responsive WAP mouse mammary tumor models have similar limitations as MMTV transgenic mice. WAP promoter expression is mammary gland selective; however, it also has some activity in other tissues (165). Furthermore, WAP models are limited in their ability to activate transgene expression in all mammary epithelial cell types, possibly contributing to the strictly luminal molecular tumor profiles observed in WAP transgenic mice (51,71). Therefore, WAP mammary tumor models do not recapitulate the tumor diversity observed in breast cancer (71). However, expression of different oncogenes using the WAP promoter does demonstrate that distinct oncogenes influence tumor subtype.

BRCA transgenic mouse mammary tumor models

As discussed in an earlier section, BRCA1 and 2 proteins are critical components of DNA repair machinery (89,105), and mutations in these genes are associated with high rates of familial breast and ovarian cancers (166). In addition, breast tumors harboring BRCA mutations are typically Basal-like and have a poor prognosis (87,88). Therefore, several transgenic mouse models have been developed to study BRCA mutations in the mouse mammary gland (117,167).

Initially, investigators observed that germline deletion of BRCA1 results in embryonic lethality due to lack of sufficient cellular proliferation (168-170); therefore, LoxP-flanked conditional BRCA1 knockout mice were generated (171-175). Deletion of BRCA1 in the mammary epithelium was then achieved by breeding BRCA1 conditional knockouts with WAP and MMTV-Cre mice; however, it was found that additional mutations in p53 were necessary for

efficient tumor growth (175,176). These results demonstrated that other mutations in tumor suppressors might be necessary to drive BRCA1-mutant breast cancer progression.

Also, since BRCA1-mutant breast cancers have a basal molecular profile (87), it was hypothesized that the basal progenitor population may be the tumor cell of origin for these cancers. To test this, the basal K14 promoter was used to target BRCA1 and p53 loss to the basal mammary epithelium. The resulting tumors closely modeled the human disease counterpart with their high proliferation profiles, poor histological grades, ER loss, and genomic instability (171). These results suggested that BRCA1-mutant Basal-like breast cancers originate from basal cells.

Conversely, a more recent study demonstrated that the luminal progenitor population is the tumor cell of origin for BRCA1-mutant Basal-like breast cancer. In this study, Molyneux et al. used cell-specific Cre drivers to delete BRCA1 in mouse luminal or basal mammary progenitors. The histologic and molecular profiles of luminal cell-derived tumors closely resembled Basal-like breast cancers. Basal progenitors, on the other hand, gave rise to myoepitheliomas, which are rare in breast cancer (117). This study was different from the preceding reports, in that BRCA1 was deleted in a p53 heterozygous background. Molyneux et al. argue that there is cooperation between complete loss of p53 and BRCA deletion in previously reported models, resulting in a Basal-like tumor phenotype originating from basal progenitors (171,177); however, the p53 heterozygous background may be a more accurate model of BRCA-mutant Basal-like breast cancer. As discussed in a previous section, analyses of primary preneoplastic breast tissues support the hypothesis that the luminal progenitor is the cell of origin for BRCA-mutant Basal-like breast cancer (115). However, since mammary epithelial stem cell and progenitor compartments are heterogeneous (38), further studies need to be performed to assess how many different populations have the capacity to give rise to BRCA-mutant Basal-like breast cancer. Furthermore, p53 loss of function is highly associated with Basal-like breast cancer (89); therefore, potential cooperation between p53 and BRCA mutations in breast cancer should not be ruled out.

DMBA mouse mammary tumors

7, 12-Dimethylbenz(a)anthracene (DMBA) is a polycyclic aromatic hydrocarbon that has been used as a carcinogen in many laboratory animals (178). DMBA interacts with and activates the cytosolic aryl hydrocarbon receptor (AHR); this leads to AHR cofactor binding and translocation into the nucleus (179,180). In turn, AHR acts as a transcription factor that promotes the expression of genes that metabolize DMBA into DNA binding compounds that lead to DNA damage and cellular transformation (181,182). In addition, AHR activity has been shown to regulate mechanisms of cellular proliferation and survival, which may further contribute to tumor growth (183-187). Therefore, administration of DMBA to mice can be used to generate mammary tumors (178).

About 75% of mice treated with DMBA develop mammary tumors; however, tumors in other tissues such as lungs, skin, and blood can also occur in 5-30% of mice. Therefore, care needs to be taken when examining metastases. Histologically, the DMBA-induced mammary tumors are fairly metaplastic and primarily squamous, but tubular, spindloid, papillary, and acinar tumors can also form (188). In addition to this histologic diversity, the molecular profiles of DMBA tumors are heterogeneous, but most tumors fall into the basal subtype (71). Due to this tumor diversity, this model does represent some of the heterogeneity observed in breast cancer; however, factors that drive tumor propagation can vary.

Transplantation mouse mammary tumor models

As discussed in a previous section, the major mammary gland developmental events take place after puberty (1). Because of this, the mammary fat pad can be “cleared” of epithelial cells prior to puberty, and transplanted primary mammary cells can be used to regenerate functional ductal trees (189). Taking advantage of this, Welm et al. demonstrated that gene expression could be specifically targeted to the mammary epithelium by lentiviral infection of primary cells prior to transplantation (190).

Smith et al. used this lentiviral infection and transplantation approach to express the PYMT oncogene in all mammary epithelial cell populations. Interestingly, this study demonstrated

that PYMT can give rise to molecularly and histologically diverse tumor types, recapitulating the disease heterogeneity observed in breast cancer. Unlike the MMTV-PYMT tumor model, the lentiviral approach demonstrated that expressing the same oncogene in diverse populations of cells results in diverse tumor phenotypes (191). This suggests that the tumor cell of origin may play a key role in influencing tumor subtype.

Summary

Overall, transgenic mouse mammary tumor models are limited in their ability to recapitulate the broad molecular and histological heterogeneity observed in breast cancer (71). However, transgenic mice such as MMTV-PYMT and MMTV-Wnt do demonstrate that expression of different oncogenes under the control the same promoter can lead to the development to distinct tumor types (71). Therefore, oncogenes and mutations may play a role in defining breast cancer phenotype. Despite these findings, it is still unclear whether the tumor cell of origin can also influence tumor subtype. Unfortunately, current transgenic mouse mammary tumor models cannot be used to target oncogene expression to the full spectrum of mammary epithelial cell types, in a cell lineage restricted context. Due to this limitation, the purpose of the work described in this dissertation was to develop new methodologies that could test the tumor cell of origin hypothesis.

The following chapters discuss a series of alternative approaches to transgenic mouse mammary tumor models, which utilize lentiviral infection and primary cell transplantation techniques to transform specific mouse mammary epithelial cell populations. Using these lentiviral infection and transplantation techniques, we were able to test whether the transformation of specific mammary epithelial cell populations influences breast cancer subtype.

References

1. Macias H, Hinck L. Mammary gland development. Wiley Interdiscip Rev Dev Biol 2012;1(4):533-57.

2. Balinsky BI. On the prenatal growth of the mammary gland rudiment in the mouse. J Anat 1950;84(3):227-35.

3. Propper AY. Wandering epithelial cells in the rabbit embryo milk line. A preliminary scanning electron microscope study. Dev Biol 1978;67(1):225-31.

4. Cowin P, Wysolmerski J. Molecular mechanisms guiding embryonic mammary gland development. Cold Spring Harb Perspect Biol 2010;2(6):a003251.

5. Hogg NA, Harrison CJ, Tickle C. Lumen formation in the developing mouse mammary gland. J Embryol Exp Morphol 1983;73:39-57.

6. Sakakura T, Sakagami Y, Nishizuka Y. Dual origin of mesenchymal tissues participating in mouse mammary gland embryogenesis. Dev Biol 1982;91(1):202-7.

7. Markadieu N, Crutzen R, Blero D, Erneux C, Beauwens R. Hydrogen peroxide and epidermal growth factor activate phosphatidylinositol 3-kinase and increase sodium transport in A6 cell monolayers. Am J Physiol Renal Physiol 2005;288(6):F1201-12.

8. Ruan W, Kleinberg DL. Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. Endocrinology 1999;140(11):5075-81.

9. Daniel CW, Silberstein GB, Strickland P. Direct action of 17 beta-estradiol on mouse mammary ducts analyzed by sustained release implants and steroid autoradiography. Cancer Res 1987;47(22):6052-7.

10. Silberstein GB, Van Horn K, Shyamala G, Daniel CW. Essential role of endogenous estrogen in directly stimulating mammary growth demonstrated by implants containing pure antiestrogens. Endocrinology 1994;134(1):84-90.

11. Williams JM, Daniel CW. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev Biol 1983;97(2):274-90.

12. Brisken C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA. A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc Natl Acad Sci U S A 1998;95(9):5076-81.

13. Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, et al. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. EMBO J 1997;16(23):6926-35.

14. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 1995;9(18):2266-78.

15. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, et al. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev 1997;11(2):167-78.

16. Vomachka AJ, Pratt SL, Lockefeer JA, Horseman ND. Prolactin gene-disruption arrests mammary gland development and retards T-antigen-induced tumor growth. Oncogene 2000;19(8):1077-84.

17. Baxter FO, Came PJ, Abell K, Kedjouar B, Huth M, Rajewsky K, et al. IKKbeta/2 induces TWEAK and apoptosis in mammary epithelial cells. Development 2006;133(17):3485-94.

18. Marti A, Ritter PM, Jager R, Lazar H, Baltzer A, Schenkel J, et al. Mouse mammary gland involution is associated with cytochrome c release and caspase activation. Mech Dev 2001;104(1-2):89-98.

19. Song J, Sapi E, Brown W, Nilsen J, Tartaro K, Kacinski BM, et al. Roles of Fas and Fas ligand during mammary gland remodeling. J Clin Invest 2000;106(10):1209-20.

20. Balogh GA, Heulings R, Mailo DA, Russo PA, Sheriff F, Russo IH, et al. Genomic signature induced by pregnancy in the human breast. Int J Oncol 2006;28(2):399-410.

21. D'Cruz CM, Moody SE, Master SR, Hartman JL, Keiper EA, Imielinski MB, et al. Persistent parity-induced changes in growth factors, TGF-beta3, and differentiation in the rodent mammary gland. Mol Endocrinol 2002;16(9):2034-51.

22. Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, et al. Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc Natl Acad Sci U S A 1997;94(7):3425-30.

23. Visvader JE. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev 2009;23(22):2563-77.

24. Daniel CW, De Ome KB, Young JT, Blair PB, Faulkin LJ, Jr. The in vivo life span of normal and preneoplastic mouse mammary glands: a serial transplantation study. Proc Natl Acad Sci U S A 1968;61(1):53-60.

25. Hoshino K. Morphogenesis and growth potentiality of mammary glands in mice. I. Transplantability and growth potentiality of mammary tissue of virgin mice. J Natl Cancer Inst 1962;29:835-51.

26. Smith GH, Medina D. A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. J Cell Sci 1988;90 (Pt 1):173-83.

27. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439(7072):84-8.

28. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, et al. Purification and unique properties of mammary epithelial stem cells. Nature 2006;439(7079):993-7.

29. Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ. CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res 2006;8(1):R7.

30. Shehata M, Teschendorff A, Sharp G, Novcic N, Russell IA, Avril S, et al. Phenotypic and functional characterisation of the luminal cell hierarchy of the mammary gland. Breast Cancer Res 2012;14(5):R134.

31. Barker N, Tan S, Clevers H. Lgr proteins in epithelial stem cell biology. Development 2013;140(12):2484-94.

32. Plaks V, Brenot A, Lawson DA, Linnemann JR, Van Kappel EC, Wong KC, et al. Lgr5-expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. Cell Rep 2013;3(1):70-8.

33. de Visser KE, Ciampricotti M, Michalak EM, Tan DW, Speksnijder EN, Hau CS, et al. Developmental stage-specific contribution of LGR5(+) cells to basal and luminal epithelial lineages in the postnatal mammary gland. J Pathol 2012;228(3):300-9.

34. Rios AC, Fu NY, Lindeman GJ, Visvader JE. In situ identification of bipotent stem cells in the mammary gland. Nature 2014;506(7488):322-7.

35. Makarem M, Kannan N, Nguyen LV, Knapp DJ, Balani S, Prater MD, et al. Developmental changes in the in vitro activated regenerative activity of primitive mammary epithelial cells. PLoS Biol 2013;11(8):e1001630.

36. Spike BT, Engle DD, Lin JC, Cheung SK, La J, Wahl GM. A mammary stem cell population identified and characterized in late embryogenesis reveals similarities to human breast cancer. Cell Stem Cell 2012;10(2):183-97.

37. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature 2011;479(7372):189-93.

38. Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 2014;28(11):1143-58.

39. Moumen M, Chiche A, Cagnet S, Petit V, Raymond K, Faraldo MM, et al. The mammary myoepithelial cell. Int J Dev Biol 2011;55(7-9):763-71.

40. Adriance MC, Inman JL, Petersen OW, Bissell MJ. Myoepithelial cells: good fences make good neighbors. Breast Cancer Res 2005;7(5):190-7.

41. Emerman JT, Vogl AW. Cell size and shape changes in the myoepithelium of the mammary gland during differentiation. Anat Rec 1986;216(3):405-15.

42. Deugnier MA, Moiseyeva EP, Thiery JP, Glukhova M. Myoepithelial cell differentiation in the developing mammary gland: progressive acquisition of smooth muscle phenotype. Dev Dyn 1995;204(2):107-17.

43. Haaksma CJ, Schwartz RJ, Tomasek JJ. Myoepithelial cell contraction and milk ejection are impaired in mammary glands of mice lacking smooth muscle alpha-actin. Biol Reprod 2011;85(1):13-21.

44. Sun P, Yuan Y, Li A, Li B, Dai X. Cytokeratin expression during mouse embryonic and early postnatal mammary gland development. Histochem Cell Biol 2010;133(2):213-21.

45. Muschler J, Streuli CH. Cell-matrix interactions in mammary gland development and breast cancer. Cold Spring Harb Perspect Biol 2010;2(10):a003202.

46. Taddei I, Deugnier MA, Faraldo MM, Petit V, Bouvard D, Medina D, et al. Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. Nat Cell Biol 2008;10(6):716-22.

47. Runswick SK, O'Hare MJ, Jones L, Streuli CH, Garrod DR. Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. Nat Cell Biol 2001;3(9):823-30.

48. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. J Cell Biol 2007;176(1):19-26.

49. Harris J, Stanford PM, Sutherland K, Oakes SR, Naylor MJ, Robertson FG, et al. Socs2 and elf5 mediate prolactin-induced mammary gland development. Mol Endocrinol 2006;20(5):1177-87.

50. Lim E, Wu D, Pal B, Bouras T, Asselin-Labat ML, Vaillant F, et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 2010;12(2):R21.

51. Wagner KU, Boulanger CA, Henry MD, Sgagias M, Hennighausen L, Smith GH. An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. Development 2002;129(6):1377-86.

52. Chang TH, Kunasegaran K, Tarulli GA, De Silva D, Voorhoeve PM, Pietersen AM. New insights into lineage restriction of mammary gland epithelium using parity-identified mammary epithelial cells. Breast Cancer Res 2014;16(1):R1.

53. Andrechek ER, White D, Muller WJ. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene 2005;24(5):932-7.

54. Lafkas D, Rodilla V, Huyghe M, Mourao L, Kiaris H, Fre S. Notch3 marks clonogenic mammary luminal progenitor cells in vivo. J Cell Biol 2013;203(1):47-56.

55. Guo W, Keckesova Z, Donaher JL, Shibue T, Tischler V, Reinhardt F, et al. Slug and Sox9 cooperatively determine the mammary stem cell state. Cell 2012;148(5):1015-28.

56. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133(4):704-15.

57. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 1999;398(6729):708-13.

58. Nassour M, Idoux-Gillet Y, Selmi A, Come C, Faraldo ML, Deugnier MA, et al. Slug controls stem/progenitor cell growth dynamics during mammary gland morphogenesis. PLoS One 2012;7(12):e53498.

59. Yalcin-Ozuysal O, Fiche M, Guitierrez M, Wagner KU, Raffoul W, Brisken C. Antagonistic roles of Notch and p63 in controlling mammary epithelial cell fates. Cell Death Differ 2010;17(10):1600-12.

60. Bouras T, Pal B, Vaillant F, Harburg G, Asselin-Labat ML, Oakes SR, et al. Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. Cell Stem Cell 2008;3(4):429-41.

61. Raouf A, Zhao Y, To K, Stingl J, Delaney A, Barbara M, et al. Transcriptome analysis of the normal human mammary cell commitment and differentiation process. Cell Stem Cell 2008;3(1):109-18.

62. Oakes SR, Naylor MJ, Asselin-Labat ML, Blazek KD, Gardiner-Garden M, Hilton HN, et al. The Ets transcription factor Elf5 specifies mammary alveolar cell fate. Genes Dev 2008;22(5):581-6.

63. Chakrabarti R, Hwang J, Andres Blanco M, Wei Y, Lukacisin M, Romano RA, et al. Elf5 inhibits the epithelial-mesenchymal transition in mammary gland development and breast

cancer metastasis by transcriptionally repressing Snail2. Nat Cell Biol 2012;14(11):1212-22.

64. Chakrabarti R, Wei Y, Romano RA, DeCoste C, Kang Y, Sinha S. Elf5 regulates mammary gland stem/progenitor cell fate by influencing notch signaling. Stem Cells 2012;30(7):1496-508.

65. Yamaji D, Na R, Feuermann Y, Pechhold S, Chen W, Robinson GW, et al. Development of mammary luminal progenitor cells is controlled by the transcription factor STAT5A. Genes Dev 2009;23(20):2382-7.

66. Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z. GATA-3 maintains the differentiation of the luminal cell fate in the mammary gland. Cell 2006;127(5):1041-55.

67. Eeckhoute J, Keeton EK, Lupien M, Krum SA, Carroll JS, Brown M. Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. Cancer Res 2007;67(13):6477-83.

68. Asselin-Labat ML, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, et al. Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. Nat Cell Biol 2007;9(2):201-9.

69. Siegel PM, Muller WJ. Transcription factor regulatory networks in mammary epithelial development and tumorigenesis. Oncogene 2010;29(19):2753-9.

70. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12(5):R68.

71. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 2007;8(5):R76.

72. Viale G. The current state of breast cancer classification. Ann Oncol 2012;23 Suppl 10:x207-10.

73. Ellis IOS, S. J .; Sastre-garau, X.; et al. Invasive breast carcinoma. In: Tavassoli FD, P., editor. Tumours of the Breast and Female Genital Organs. Lyon: IaRC Press; 2003.

74. Pestalozzi BC, Zahrieh D, Mallon E, Gusterson BA, Price KN, Gelber RD, et al. Distinct clinical and prognostic features of infiltrating lobular carcinoma of the breast: combined results of 15 International Breast Cancer Study Group clinical trials. J Clin Oncol 2008;26(18):3006-14.

75. Viale G, Rotmensz N, Maisonneuve P, Orvieto E, Maiorano E, Galimberti V, et al. Lack of prognostic significance of "classic" lobular breast carcinoma: a matched, single institution series. Breast Cancer Res Treat 2009;117(1):211-4.

76. Arpino G, Clark GM, Mohsin S, Bardou VJ, Elledge RM. Adenoid cystic carcinoma of the breast: molecular markers, treatment, and clinical outcome. Cancer 2002;94(8):2119-27.

77. Colleoni M, Rotmensz N, Maisonneuve P, Mastropasqua MG, Luini A, Veronesi P, et al. Outcome of special types of luminal breast cancer. Ann Oncol 2012;23(6):1428-36.

78. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: how special are they? Mol Oncol 2010;4(3):192-208.

79. Orvieto E, Maiorano E, Bottiglieri L, Maisonneuve P, Rotmensz N, Galimberti V, et al. Clinicopathologic characteristics of invasive lobular carcinoma of the breast: results of an analysis of 530 cases from a single institution. Cancer 2008;113(7):1511-20.

80. de Azambuja E, Cardoso F, de Castro G, Jr., Colozza M, Mano MS, Durbecq V, et al. Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients. Br J Cancer 2007;96(10):1504-13.

81. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983;31(1):13-20.

82. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ, et al. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann Oncol 2009;20(8):1319-29.

83. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol 2010;28(16):2784-95.

84. Thor AD, Liu S, Moore DH, 2nd, Edgerton SM. Comparison of mitotic index, in vitro bromodeoxyuridine labeling, and MIB-1 assays to quantitate proliferation in breast cancer. J Clin Oncol 1999;17(2):470-7.

85. Carlson RW, Moench SJ, Hammond ME, Perez EA, Burstein HJ, Allred DC, et al. HER2 testing in breast cancer: NCCN Task Force report and recommendations. J Natl Compr Canc Netw 2006;4 Suppl 3:S1-22; quiz S23-4.

86. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007;25(1):118-45.

87. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406(6797):747-52.

88. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98(19):10869-74.

89. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature 2012;490(7418):61-70.

90. Zhang MH, Man HT, Zhao XD, Dong N, Ma SL. Estrogen receptor-positive breast cancer molecular signatures and therapeutic potentials (Review). Biomed Rep 2014;2(1):41-52.

91. Deisenroth C, Thorner AR, Enomoto T, Perou CM, Zhang Y. Mitochondrial Hep27 is a c-Myb target gene that inhibits Mdm2 and stabilizes p53. Mol Cell Biol 2010;30(16):3981-93.

92. Ades F, Zardavas D, Bozovic-Spasojevic I, Pugliano L, Fumagalli D, de Azambuja E, et al. Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. J Clin Oncol 2014;32(25):2794-803.

93. Allred DC, Brown P, Medina D. The origins of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer. Breast Cancer Res 2004;6(6):240-5.

94. Osborne CK. Steroid hormone receptors in breast cancer management. Breast Cancer Res Treat 1998;51(3):227-38.

95. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med 2012;366(6):520-9.

96. Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, et al. Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study. Clin Cancer Res 2011;17(5):1147-59.

97. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. Curr Opin Cell Biol 2009;21(2):177-84.

98. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000;19(13):3159-67.

99. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 2006;355(26):2733-43.

100. Joensuu H, Kellokumpu-Lehtinen PL, Bono P, Alanko T, Kataja V, Asola R, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. N Engl J Med 2006;354(8):809-20.

101. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353(16):1659-72.

102. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 2005;353(16):1673-84.

103. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001;344(11):783-92.

104. Rexer BN, Arteaga CL. Intrinsic and acquired resistance to HER2-targeted therapies in HER2 gene-amplified breast cancer: mechanisms and clinical implications. Crit Rev Oncog 2012;17(1):1-16.

105. Welcsh PL, King MC. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. Hum Mol Genet 2001;10(7):705-13.

106. Visvader JE. Cells of origin in cancer. Nature 2011;469(7330):314-22.

107. Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN. Chronic myelocytic leukemia. Origin of some lymphocytes from leukemic stem cells. J Clin Invest 1978;62(4):815-23.

108. Huntly BJ, Shigematsu H, Deguchi K, Lee BH, Mizuno S, Duclos N, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell 2004;6(6):587-96.

109. Akala OO, Park IK, Qian D, Pihalja M, Becker MW, Clarke MF. Long-term haematopoietic reconstitution by Trp53-/-p16Ink4a-/-p19Arf-/- multipotent progenitors. Nature 2008;453(7192):228-32.

110. Barker N, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. Cell Stem Cell 2010;6(1):25-36.

111. Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 2009;457(7229):608-11.

112. Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 2008;40(7):915-20.

113. Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, et al. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 2009;457(7229):603-7.

114. Blanpain C. Tracing the cellular origin of cancer. Nat Cell Biol 2013;15(2):126-34.

115. Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 2009;15(8):907-13.

116. Ince TA, Richardson AL, Bell GW, Saitoh M, Godar S, Karnoub AE, et al. Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 2007;12(2):160-70.

117. Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R, et al. BRCA1 Basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. Cell Stem Cell 2010;7(3):403-17.

118. Allred DC, Medina D. The relevance of mouse models to understanding the development and progression of human breast cancer. J Mammary Gland Biol Neoplasia 2008;13(3):279-88.

119. Callahan R, Smith GH. MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways. Oncogene 2000;19(8):992-1001.

120. Callahan R, Smith GH. Common integration sites for MMTV in viral induced mouse mammary tumors. J Mammary Gland Biol Neoplasia 2008;13(3):309-21.

121. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 1982;31(1):99-109.

122. Cato AC, Henderson D, Ponta H. The hormone response element of the mouse mammary tumour virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J 1987;6(2):363-8.

123. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 1987;49(4):465-75.

124. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell 1988;54(1):105-15.

125. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12(3):954-61.

126. Israel MA, Chan HW, Hourihan SL, Rowe WP, Martin MA. Biological activity of polyoma viral DNA in mice and hamsters. J Virol 1979;29(3):990-6.

127. Rodriguez-Viciana P, Collins C, Fried M. Polyoma and SV40 proteins differentially regulate PP2A to activate distinct cellular signaling pathways involved in growth control. Proc Natl Acad Sci U S A 2006;103(51):19290-5.

128. Fluck MM, Schaffhausen BS. Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. Microbiol Mol Biol Rev 2009;73(3):542-63, Table of Contents.

129. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol 2003;163(5):2113-26.

130. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. Cell 1989;57(6):931-6.

131. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A 1992;89(22):10578-82.

132. Siegel PM, Dankort DL, Hardy WR, Muller WJ. Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors. Mol Cell Biol 1994;14(11):7068-77.

133. Siegel PM, Muller WJ. Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. Proc Natl Acad Sci U S A 1996;93(17):8878-83.

134. Siegel PM, Ryan ED, Cardiff RD, Muller WJ. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J 1999;18(8):2149-64.

135. Kwong KY, Hung MC. A novel splice variant of HER2 with increased transformation activity. Mol Carcinog 1998;23(2):62-8.

136. Park JW, Neve RM, Szollosi J, Benz CC. Unraveling the biologic and clinical complexities of HER2. Clin Breast Cancer 2008;8(5):392-401.

137. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235(4785):177-82.

138. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244(4905):707-12.

139. Li Y, Hively WP, Varmus HE. Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. Oncogene 2000;19(8):1002-9.

140. Dickson C, Fantl V. Fgf-3, an oncogene in murine breast cancer. Cancer Treat Res 1994;71:331-43.

141. Nusse R. The int genes in mammary tumorigenesis and in normal development. Trends Genet 1988;4(10):291-5.

142. Shackleford GM, MacArthur CA, Kwan HC, Varmus HE. Mouse mammary tumor virus infection accelerates mammary carcinogenesis in Wnt-1 transgenic mice by insertional activation of int-2/Fgf-3 and hst/Fgf-4. Proc Natl Acad Sci U S A 1993;90(2):740-4.

143. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 1988;55(4):619-25.

144. Howe LR, Brown AM. Wnt signaling and breast cancer. Cancer Biol Ther 2004;3(1):36-41.

145. Klarmann GJ, Decker A, Farrar WL. Epigenetic gene silencing in the Wnt pathway in breast cancer. Epigenetics 2008;3(2):59-63.

146. Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, et al. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc Natl Acad Sci U S A 2000;97(8):4262-6.

147. Ryo A, Nakamura M, Wulf G, Liou YC, Lu KP. Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. Nat Cell Biol 2001;3(9):793-801.

148. Choi YW, Henrard D, Lee I, Ross SR. The mouse mammary tumor virus long terminal repeat directs expression in epithelial and lymphoid cells of different tissues in transgenic mice. J Virol 1987;61(10):3013-9.

149. Henrard D, Ross SR. Endogenous mouse mammary tumor virus is expressed in several organs in addition to the lactating mammary gland. J Virol 1988;62(8):3046-9.

150. Hobbs AA, Richards DA, Kessler DJ, Rosen JM. Complex hormonal regulation of rat casein gene expression. J Biol Chem 1982;257(7):3598-605.

151. Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 1999;19(1):1-11.

152. Davis AC, Wims M, Spotts GD, Hann SR, Bradley A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev 1993;7(4):671-82.

153. Berns EM, Foekens JA, van Putten WL, van Staveren IL, Portengen H, de Koning WC, et al. Prognostic factors in human primary breast cancer: comparison of c-myc and HER2/neu amplification. J Steroid Biochem Mol Biol 1992;43(1-3):13-9.

154. Bieche I, Lidereau R. Genetic alterations in breast cancer. Genes Chromosomes Cancer 1995;14(4):227-51.

155. Castaldo G, Oriani G, Cimino L, Topa M, Budillon G, Salvatore F, et al. Discriminant function based on serum analytes differentiates hepatocarcinoma from secondary liver neoplasia. Clin Chem 1995;41(3):439-43.

156. Cole MD. The myc oncogene: its role in transformation and differentiation. Annu Rev Genet 1986;20:361-84.

157. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme MH, Gest J, et al. Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas. Proc Natl Acad Sci U S A 1986;83(13):4834-8.

158. Schoenenberger CA, Andres AC, Groner B, van der Valk M, LeMeur M, Gerlinger P. Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. EMBO J 1988;7(1):169-75.

159. Gallahan D, Jhappan C, Robinson G, Hennighausen L, Sharp R, Kordon E, et al. Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer Res 1996;56(8):1775-85.

160. Li M, Lewis B, Capuco AV, Laucirica R, Furth PA. WAP-TAg transgenic mice and the study of dysregulated cell survival, proliferation, and mutation during breast carcinogenesis. Oncogene 2000;19(8):1010-9.

161. Gallahan D, Callahan R. Mammary tumorigenesis in feral mice: identification of a new int locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. J Virol 1987;61(1):66-74.

162. Dyson N, Buchkovich K, Whyte P, Harlow E. The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. Cell 1989;58(2):249-55.

163. Mietz JA, Unger T, Huibregtse JM, Howley PM. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. EMBO J 1992;11(13):5013-20.

164. Husler MR, Kotopoulis KA, Sundberg JP, Tennent BJ, Kunig SV, Knowles BB. Lactation-induced WAP-SV40 Tag transgene expression in C57BL/6J mice leads to mammary carcinoma. Transgenic Res 1998;7(4):253-63.

165. Wen J, Kawamata Y, Tojo H, Tanaka S, Tachi C. Expression of whey acidic protein (WAP) genes in tissues other than the mammary gland in normal and transgenic mice expressing mWAP/hGH fusion gene. Mol Reprod Dev 1995;41(4):399-406.

166. Petrucelli N, Daly MB, Feldman GL. Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Genet Med 2010;12(5):245-59.

167. Drost RM, Jonkers J. Preclinical mouse models for BRCA1-associated breast cancer. Br J Cancer 2009;101(10):1651-7.

168. Jonkers J, Berns A. Conditional mouse models of sporadic cancer. Nat Rev Cancer 2002;2(4):251-65.

169. Evers B, Jonkers J. Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects. Oncogene 2006;25(43):5885-97.

170. Kuschel B, Gayther SA, Easton DF, Ponder BA, Pharoah PD. Apparent human BRCA1 knockout caused by mispriming during polymerase chain reaction: implications for genetic testing. Genes Chromosomes Cancer 2001;31(1):96-8.

171. Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A, et al. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated Basal-like breast cancer. Proc Natl Acad Sci U S A 2007;104(29):12111-6.

172. Mak TW, Hakem A, McPherson JP, Shehabeldin A, Zablocki E, Migon E, et al. Brca1 required for T cell lineage development but not TCR loci rearrangement. Nat Immunol 2000;1(1):77-82.

173. McCarthy A, Savage K, Gabriel A, Naceur C, Reis-Filho JS, Ashworth A. A mouse model of Basal-like breast carcinoma with metaplastic elements. J Pathol 2007;211(4):389-98.

174. Shakya R, Szabolcs M, McCarthy E, Ospina E, Basso K, Nandula S, et al. The Basal-like mammary carcinomas induced by Brca1 or Bard1 inactivation implicate the BRCA1/BARD1 heterodimer in tumor suppression. Proc Natl Acad Sci U S A 2008;105(19):7040-5.

175. Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, et al. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 1999;22(1):37-43.

176. Brodie SG, Xu X, Qiao W, Li WM, Cao L, Deng CX. Multiple genetic changes are associated with mammary tumorigenesis in Brca1 conditional knockout mice. Oncogene 2001;20(51):7514-23.

177. Smart CE, Clarke C, Brooks KM, Raghavendra A, Brewster BL, French JD, et al. Targeted disruption of Brca1 in restricted compartments of the mouse mammary epithelia. Breast Cancer Res Treat 2008;112(2):237-41.

178. Medina D. Mammary tumorigenesis in chemical carcinogen-treated mice. I. Incidence in BALB-c and C57BL mice. J Natl Cancer Inst 1974;53(1):213-21.

179. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 2003;43:309-34.

180. Swanson HI, Chan WK, Bradfield CA. DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. J Biol Chem 1995;270(44):26292-302.

181. Nebert DW, Petersen DD, Fornace AJ, Jr. Cellular responses to oxidative stress: the [Ah] gene battery as a paradigm. Environ Health Perspect 1990;88:13-25.

182. Rundle A, Tang D, Hibshoosh H, Estabrook A, Schnabel F, Cao W, et al. The relationship between genetic damage from polycyclic aromatic hydrocarbons in breast tissue and breast cancer. Carcinogenesis 2000;21(7):1281-9.

183. Abdelrahim M, Smith R, 3rd, Safe S. Aryl hydrocarbon receptor gene silencing with small inhibitory RNA differentially modulates Ah-responsiveness in MCF-7 and HepG2 cancer cells. Mol Pharmacol 2003;63(6):1373-81.

184. Ge NL, Elferink CJ. A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle. J Biol Chem 1998;273(35):22708-13.

185. Holcomb M, Safe S. Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumor growth by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Cancer Lett 1994;82(1):43-7.

186. Ma Q, Whitlock JP, Jr. The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. Mol Cell Biol 1996;16(5):2144-50.

187. Puga A, Barnes SJ, Dalton TP, Chang C, Knudsen ES, Maier MA. Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. J Biol Chem 2000;275(4):2943-50.

188. Currier N, Solomon SE, Demicco EG, Chang DL, Farago M, Ying H, et al. Oncogenic signaling pathways activated in DMBA-induced mouse mammary tumors. Toxicol Pathol 2005;33(6):726-37.

189. Smith BA, Welm AL, Welm BE. On the shoulders of giants: a historical perspective of unique experimental methods in mammary gland research. Semin Cell Dev Biol 2012;23(5):583-90.

190. Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z. Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. Cell Stem Cell 2008;2(1):90-102.

191. Smith BA, Shelton DN, Kieffer C, Milash B, Usary J, Perou CM, et al. Targeting the PyMT Oncogene to Diverse Mammary Cell Populations Enhances Tumor Heterogeneity and Generates Rare Breast Cancer Subtypes. Genes Cancer 2012;3(9-10):550-63.

Figure 1.1. Mammary gland development. The mammary epithelium develops during several key postembryonic stages, including puberty, pregnancy, and lactation. Specific growth factors and hormones regulate the initiation and progression of each mammary epithelial developmental stage. This figure was adapted from Macias and Hinck, 2012 (1).

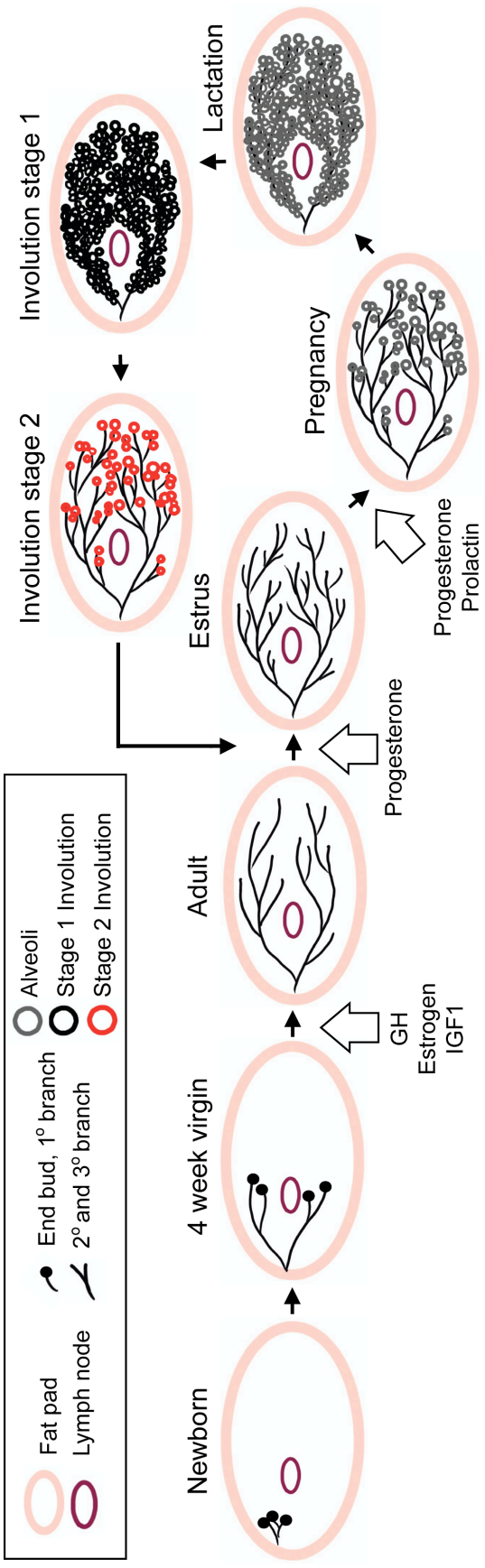
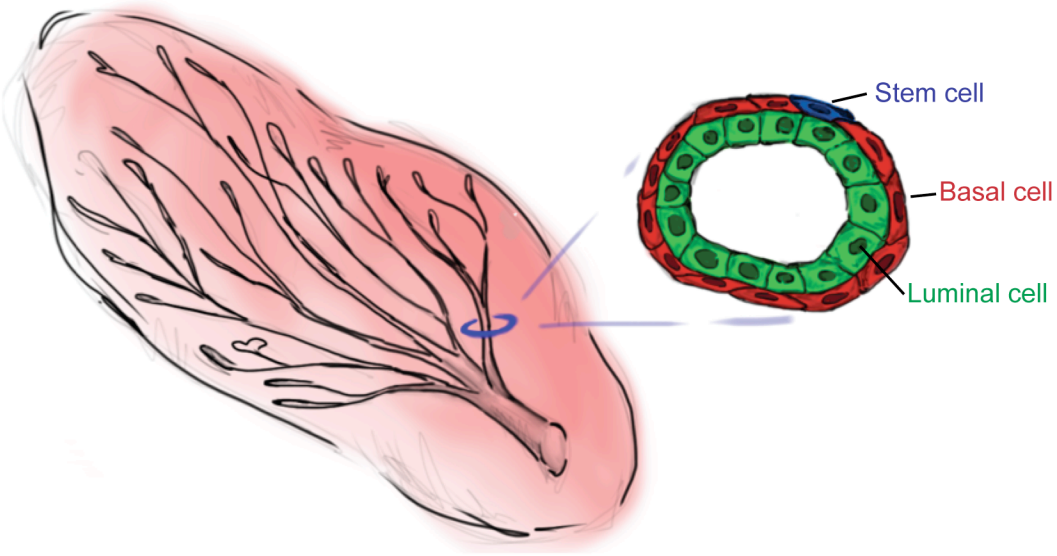

Fat pad
Lymph node
Alveoli
End bud, 1° branch
2° and 3° branch
Stage 1 Involution
Stage 2 Involution
Newborn
4 week virgin
Adult
GH
Estrogen
IGF1
Progesterone
Estrus
Involution stage 2
Involution stage 1
Progesterone
Prolactin
Pregnancy
Lactation

Figure 1.2. Mouse mammary gland epithelial cell organization and hierarchy. A. The mouse mammary gland is composed of a branched epithelial ductal network surrounded by a fat pad. The ducts are bilayered, consisting of luminal, basal, and stem cell populations. B. The mammary epithelial cell hierarchy consists of fetal and adult stem cells, lineage committed progenitors, and differentiated basal and luminal cells (adapted from Visvader and Stingl, 2014 (38)).

A. Mouse mammary gland Duct cross section



B.

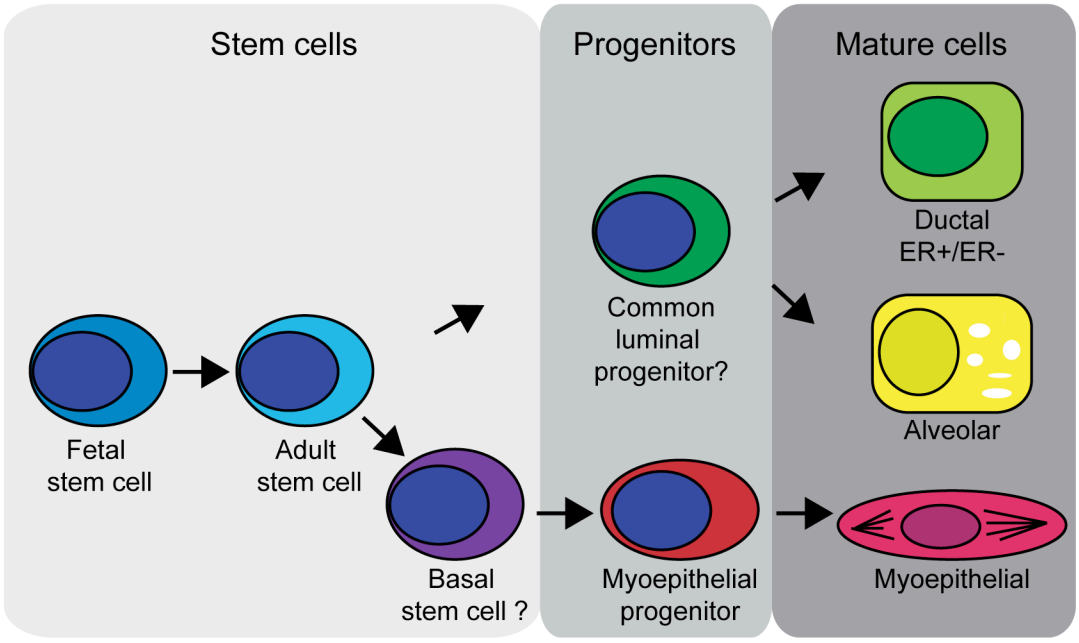


Figure 1.3. The molecular and pathophysiological heterogeneity observed in breast cancer may be explained by the genetic mutation and the tumor cell of origin hypotheses. A. The genetic mutation hypothesis proposes that different oncogenic evens in the same population of cells may contribute to different tumor phenotypes. B. The tumor cell of origin hypothesis proposes that the intrinsic properties of the initially transformed cell population may influence tumor phenotype. This figure is adapted from adapted from Visvader, 2011 (106).

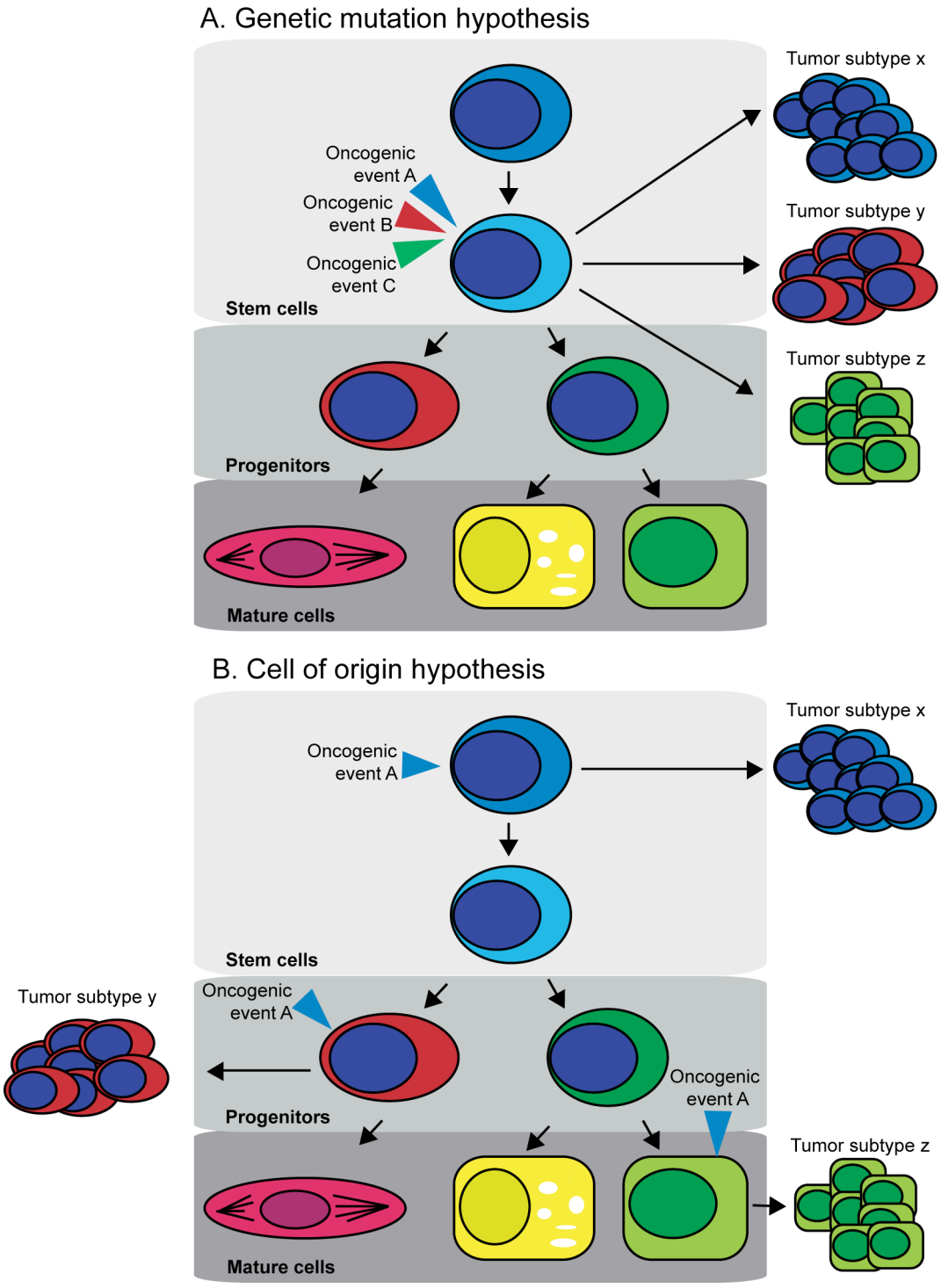
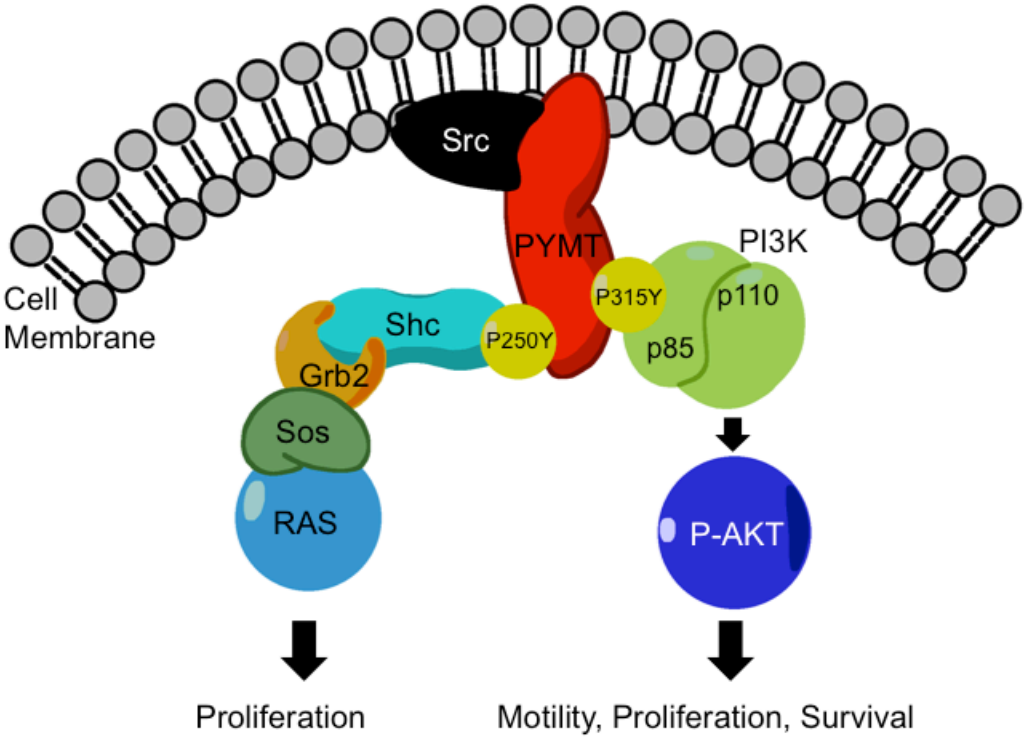

A. Genetic mutation hypothesis
Tumor subtype x
Oncogenic event A
Oncogenic event B
Oncogenic event C
Tumor subtype y
Stem cells
Tumor subtype z
Progenitors
Mature cells
B. Cell of origin hypothesis
Tumor subtype x
Oncogenic event A
Stem cells
Tumor subtype y
Oncogenic event A
Oncogenic event A
Progenitors
Tumor subtype z
Mature cells

Figure 1.4. PYMT drives tumorigenesis by acting as a membrane-anchored scaffold for activation of RAS and AKT signaling pathways. First, Src family tyrosine kinases phosphorylate PYMT at tyrosines 250 and 315. This creates binding sites for upstream components of RAS and AKT pathways. In turn, activation of RAS and AKT leads to rapid cellular transformation and tumor growth. This figure is adapted from Rodriguez-Viciana et al., 2006 (127).


Src
PYMT
PI3K
Cell
Membrane
P315Y
p110
Shc
P250Y
p85
Grb2
Sos
RAS
P-AKT
Proliferation
Motility, Proliferation, Survival

CHAPTER II

TRANSFORMATION OF ENRICHED MAMMARY CELL POPULATIONS WITH POLYOMA MIDDLE T ANTIGEN INFLUENCES TUMOR SUBTYPE AND METASTATIC POTENTIAL

This manuscript is currently under review at:

Breast Cancer Research

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The purpose of this manuscript was to test whether transformation of enriched mouse mammary epithelial cell populations influences tumor subtype and pathophysiology. This was achieved by combining lentiviral infection, fluorescence activated cell sorting, and transplantation techniques. Our results demonstrated that expression of the Polyoma Middle T Antigen oncogene within different mammary epithelial cell populations influences tumor histology, molecular subtype, and metastatic potential.

Abstract

Introduction

Breast cancer is a multifaceted disease, exhibiting significant molecular, histological, and pathological diversity. Factors that impact this heterogeneity are poorly understood; however, transformation of distinct normal cell populations of the breast may generate different tumor phenotypes. Our previous study demonstrates that the polyomavirus middle T antigen (PYMT) oncogene can establish diverse tumor subtypes when broadly expressed within mouse mammary epithelial cells. Herein, we assess the molecular, histological, and metastatic outcomes from distinct mammary cell populations transformed with PYMT.

Methods

Isolated mouse mammary epithelial cells were transduced with a lentivirus expressing PYMT and sorted into hormone receptor positive luminal (CD133+), hormone receptor negative luminal (CD133-), basal, and stem cell populations using the cell surface markers CD24, CD49f, and CD133. Each population was subsequently transplanted into syngeneic cleared mouse mammary fat pads. Tumors were classified by histology, estrogen receptor status, molecular subtype, and metastatic potential to investigate whether transformation of different enriched populations affects tumor phenotype.

Results

While enriched mammary epithelial cell populations showed no difference in the ability to form tumors or tumor latency, differences in prevalence of solid adenocarcinomas, squamous, papillary, and sebaceous-like tumors were observed. Interestingly, both molecularly Basal and Luminal tumors developed from luminal CD133+, basal, and stem cell populations; however, luminal CD133- cells gave rise exclusively to molecularly Basal tumors. Tumors arising from the luminal CD133-, basal, and stem cell populations were highly metastatic; however, luminal CD133+ cells generated tumors that were significantly less metastatic, possibly due to an inability of these tumor cells to escape the primary tumor site.

Conclusion

By combining several methodologies, including lentiviral infection, cell sorting, and transplantation, we have characterized tumors arising from enriched populations of mammary epithelial cells. We have found that expression of PYMT within different cell populations influences tumor histology, molecular subtype, and metastatic potential.

Introduction

Breast cancer can be classified into several distinct molecular and histological subtypes. These classifications provide important data which help guide patient therapy and predict outcome (1,2). Understanding how different breast cancer subtypes arise, and their cellular etiology, may improve strategies for detection, treatment, and prevention of the disease.

Several studies support the concept that the tumor cell of origin plays a role in establishing the histological and molecular heterogeneity observed in breast cancer. For example, molecular markers that distinguish basal and luminal cell layers of the normal breast can also be used to differentiate Basal-like and Luminal breast cancer molecular subtypes. Tumors classified as the Luminal subtype express estrogen receptor (ER) and are generally dependent on estrogen for growth, recapitulating characteristics of a subset of normal, luminal epithelial cells found in the breast. In contrast, Basal-like breast cancer expresses molecular markers associated with basal epithelium. Furthermore, molecular signatures derived from normal mammary cell populations correlate with signatures from some tumor subtypes. For example, spindloid and claudin-low tumors exhibit molecular signatures associated with normal breast stem cells (3-5). Even differentiated mammary epithelial cells can share molecular features with cancer subtypes, as tumors histologically classified as lipid-rich carcinoma of the breast express metabolic and differentiation markers observed in alveolar cells (6-8). Given these similarities between normal and transformed cells, it is possible that transformation of distinct cell types in the mammary gland contributes to unique tumor phenotypes.

Several studies have demonstrated that cancer phenotype is coupled, to some extent, with the cellular origin of the tumor. Ince et al. used different cell culture conditions to enrich for BPEC (luminal-like) and HMEC (myoepithelial-like) human breast cell populations. Each

population was subsequently transformed with a common set of oncogenic drivers and, when transplanted, they established distinct tumor phenotypes that included differences in tumor histology and metastasis (9). In addition, disparities in tumor phenotype were observed when BRCA1 loss-of-function was targeted to luminal cells versus basal cells (10). These studies suggest that intrinsic differences between cell populations may influence the histopathology of the tumor's they generate.

The polyomavirus middle T antigen (PYMT) oncogene has been used extensively in mice to model breast cancer. In these models, PYMT drives transformation of mammary epithelial cells by signaling through pathways frequently perturbed in breast cancer, including Src, Ras, and PI3K (11-14). In MMTV-PYMT transgenic mice, the expression of PYMT is restricted to a subset of hormone-responsive mammary epithelial cells, and tumors that arise generally have features of luminal-like adenocarcinoma. This tumor phenotype may be influenced by the cell-lineage restricted expression of PYMT (3,15). In support of this, our previous work demonstrated that using a lentiviral approach to express PYMT broadly in all populations of the mammary epithelium permitted the development of molecularly and histologically diverse tumors [14]. The contrasting tumor phenotypes observed between these models may potentially be explained by differences in the cell type that expressed PYMT; a concept we explore herein.

In the work presented here, we test whether different tumor phenotypes arise when specific mammary epithelial cell (MEC) populations are transformed with the PYMT oncogene. The results demonstrate that the precursor cell type does influence the prevalence of tumor histopathology, molecular subtype, and metastatic potential.

Methods

Mice

FVB/NJ mice were obtained from The Jackson Laboratory and maintained in a pathogen-free facility. Mice were handled according to University of Utah-approved Institutional Animal Care and Use Committee procedures.

Generation of mouse mammary tumors

Freshly isolated MECs, collected from 8-10-week-old FVB/NJ mice, were infected with EF1α-PYMT-ZsGreen lentivirus overnight at 37^{o}C, as described previously (8). Following infection, cells were washed five times with Hanks Balanced Salt Solution (HBSS, Gibco) and incubated with 0.05% Trypsin-EDTA (Gibco) to isolate single cells. Trypsin was inactivated with MEC media (8), and cell clumps were removed by straining MECs through a 40μm cell strainer (Falcon). MECs were then re-suspended in wash buffer (HBSS+ 2% fetal bovine serum [FBS [HyClone]) and kept on ice for antibody staining and fluorescence activated cell sorting (FACS).

Staining consisted of 6 tubes: 1) no antibody control, 2) CD24-V450 control, 3) CD49f-PE control, 4) CD133-APC control, 5) 7AAD control, 6) CD24-V450/CD49f-PE/CD133-APC/7AAD sample. During antibody staining, control tubes contained 5 X 10^4 cells and the sample tube contained 20 X 10^6 cells re-suspended in 200ul wash buffer. All antibodies were obtained from BD Pharmingen and were used at a 1:100 dilution. After adding the primary antibodies, cells were incubated on ice for 15 minutes. Following incubation, cells were washed with 1mL of wash buffer and centrifuged at 1000g for 2 minutes. Stained MECs were then re-suspended in wash buffer and sorted into luminal CD133+, luminal CD133-, basal, and stem populations as described previously (16-19), on a FACSAria-II SORP high-speed cell sorter, using FACSDiva Version 6.1.3 software for analysis. Isolated MEC populations were kept on ice until transplantation.

For each transplantation, 1 X 10^5 untransduced and unsorted MECs were mixed with 2 X 10^4 transduced luminal CD133+, luminal CD133-, basal, or 5 X 10^3 stem-enriched MECs. MECs were then re-suspended in 10μL Matrigel (BD Biosciences) per transplantation, and the Matrigel-cell mixture was injected into the fourth cleared inguinal mammary fat pads of 3-week-old FVB/NJ mice. Tumor growth was monitored, and tumors were collected upon reaching 2 cm in diameter. Once tumors were harvested, viable cells were collected using the same protocol for MEC isolation, and then frozen in freeze media, as described previously (8). Portions of the tumors were also flash frozen for RNA isolation using the Qiagen RNeasy kit, and additional tumor fragments were processed for paraffin embedding.

Infection, cell sorting, and transplantation experiments were performed over two rounds.

Each time, 10 transplants were performed per sorted MEC population, for a total 20 transplants per group.

Antibody staining and histology

Portions of transduced and FACS-sorted MECs were used to quantify basal and luminal cell enrichment. For each isolated population 1 X 10^4 MECs, re-suspended in 200 μL wash buffer, were centrifuged (Cytospin 4 Cytocentrifuge, Thermo Scientific) onto slides (Shandon Cytoslide, Thermo Scientific) at 900 rpm, for 10 minutes. Cytospun cells were then incubated with fix (4% paraformaldehyde in phosphate buffered saline [PBS]) for 15 minutes and washed 5 times with PBS for 5 minutes each. Fixed cells were permeabilized for 10 minutes with 0.2% Triton X-100 (Sigma) in PBS, washed with 1% bovine serum albumin (BSA, EMD Chemicals) in PBS, and blocked with 1% BSA in PBS for 10 minutes. Cells were then incubated with primary antibodies against keratin 14 (K14, 1:400, rabbit, PRB-P-100, Covance) and keratin 8 (K8, 1:50, rat, Troma-I, Developmental Studies Hybridoma Bank) for 1 hour at room temperature. Following incubation, slides were washed with 1% BSA in PBS and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and secondary antibodies Alexa Fluor 594 Chicken Anti-Rat IgG (1:1000, Invitrogen) and Alexa Fluor 488 Goat Anti-Rabbit IgG (1:1000, Invitrogen).

Paraffin-embedded tumor samples were processed and analyzed by hematoxylin and eosin (H&E), ER immunohistochemistry (IHC), and cytokeratin staining, as described previously (8). The following primary antibodies were used: K14 (1:400, rabbit, PRB-P-100, Covance), K8 (1:50, rat, Troma-I, Developmental Studies Hybridoma Bank), ER (1:200, Santa Cruz Biotechnology). Secondary antibodies included Alexa Fluor 594 Chicken Anti-Rat IgG (1:1000, Invitrogen), Alexa Fluor 488 Goat Anti-Rabbit IgG (1:1000, Invitrogen), and biotin-sp-conjugated (1:1000, Jackson ImmunoResearch).

All immunofluorescent imaging was performed on an Olympus IX81 microscope using a Hamamatsu Photonics ORCA-ER camera. Fluorescent image recording and processing were performed using Slidebook 64 version 5.0.0.24. Slides processed for IHC and H&E staining were imaged on an Olympus Bx50 microscope with a Canon EOS Rebel XSI camera using EOS imaging software. Any changes in contrast and brightness were performed in Photoshop CS4

(Adobe Systems) software on entire images to enhance appearance, without altering image content.

Microarray analysis

Flash frozen tumors were randomly selected from each of the tumor groups for RNA extraction and microarray analysis. Total RNA was isolated using the Qiagen RNeasy kit. All steps of microarray processing, data filtering and normalization, and analysis were performed as described previously (8). Batch adjustment was performed in two batches: dataset generated by Herschkowitz et al. was treated as one batch (Gene Expression Omnibus series GSE3165) [3], and data generated at the Huntsman Cancer Institute (HCI) was treated as a second batch. The HCI microarray dataset has been deposited in NCBI's Gene Expression Omnibus with the accession number GSE64453.

Assessment of tumor metastasis

As described above, transplantation experiments were performed twice for each transduced and enriched MEC population. Lung metastasis for the first round was assessed by H&E staining; lung tissue processing and staining was performed as described above. Paraffin embedded lungs were serially sectioned at 10 μm and every fifth slide was stained and examined for metastasis. For the second round of transplants, lung metastases were analyzed by fluorescent imaging after flattening the lungs between two glass slides. Slides were imaged as described above, and numbers of unique metastatic sites and tumor areas were quantified using ImageJ software. Prevalence of lung metastases and numbers of metastatic foci were consistent over two rounds of transplants.

To quantify circulating tumor cell (CTC) numbers, fresh whole blood was collected by cardiac puncture immediately after mice were euthanized according to University of Utah-approved Institutional Animal Care and Use Committee procedures. CTCs were isolated for FACS analysis as described previously from mice bearing primary tumors, as well as no-tumor control mice (20). CTCs expressing ZsGreen were detected by analyzing cells using a FACScan cytometer (BD), and results were quantified using FlowJo Software (Treestar). Due to low

numbers of CTCs present within isolated whole blood, the ZsGreen-positive threshold was set at 0.05% of no-tumor control background signal. This threshold was then used as a baseline for detecting CTCs in tumor-bearing mice. All CTC values were then normalized to no-tumor control background signal.

Tail vein injections were performed to assess the ability of tumor cells to colonize the lung after introduction into the bloodstream. Single cells were isolated from primary tumors using the same methodologies for as MEC isolation. Cells were then re-suspended in HBSS at 10 X 10^6 cells/mL. 250 µL of the HBSS/cell mixture (2.5 X 10^5 cells) were injected into the lateral tail veins of 8-12-week-old FVB/NJ mice. Cells isolated from individual tumors were injected into five mice each. 20 days post injection, mice were sacrificed and tumor lung foci numbers were quantified by fluorescent imaging, using ImageJ software.

Statistical analysis

All data analyses were performed using GraphPad Prism 6.0d software. For each analysis, specific statistical tests are indicated in the figure legends. P values are denoted by asterisks in the figures which represent the following values: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.0005$, **** = $p<0.0001$.

Results

FACS-enriched mammary cell populations expressing PYMT develop tumors with equivalent latency

Using a multistep procedure (Figure 2.1A), we evaluated whether transformation of distinct, normal mammary cell populations would influence tumor progression and pathology. Freshly isolated primary MECs were infected with the EF1α-PYMT-ZsGreen lentivirus (8), and then stained and sorted for the cell surface proteins CD24, CD49f, and CD133, which are markers known to delineate luminal, basal, and stem cell populations. In accordance with published studies, luminal, basal and stem cells were isolated based on their expression of CD49f and CD24 (Figure 2.1B) (16,17,19). The luminal cell population was further separated into hormone-receptor positive and negative fractions by expression of CD133 (herein called luminal

CD133+ and luminal CD133-, respectively) (Figure 2.1B) (18). We next verified that enriched populations contained the expected keratin markers by evaluating each population for the expression of luminal cell-specific K8 and basal cell-specific K14 by immunofluorescence (Figure 2.1C) (21). As expected, at least 90% of cells within each group expressed the appropriate marker(s) (Figure 2.1D) (16-19). Interestingly, approximately 15% of luminal-CD133- cells and 33% of enriched stem cells were positive for both K8 and K14, suggesting the presence of a multipotent progenitor population within these groups (22-25). Additional experimental replicates generated similar cytokeratin expression profiles (Supplementary Figure 2.1).

To assess tumor formation and progression of each subpopulation, transduced luminal CD133+, luminal CD133-, basal, and stem cells were individually transplanted into cleared mouse mammary fat pads. Each enriched MEC population was co-transplanted with unsorted and untransduced MECs to minimize potential trans-differentiation effects (26). Tumors arose from each enriched-MEC population and no statistical difference in average tumor latency or tumor-free survival was observed (Figure 2.1E), demonstrating that all enriched MEC populations have the capacity to undergo transformation and generate tumors.

Enriched MEC populations establish tumors with broad histopathology

We next analyzed the histology of tumors originating from each of the enriched MEC populations. We classified tumors by H&E staining and cytokeratin expression, and identified the following histologies: acinar, papillary, solid adenocarcinoma, squamous, lipid-rich, and sebaceous-like (Figures 2.2A-F, Table 2.1). The frequency of a specific histology was quantified by estimating its area in 2-3 different sections per tumor (Figure 2.2G-K). Several trends became apparent. First, while papillary features were observed in all tumor origins, they were significantly enriched in tumors arising from luminal CD133+ cells as compared to the stem-enriched population. Next, the predominant histology generated by luminal CD133- cells was solid adenocarcinoma, while basal- and stem-enriched populations established more squamous tumors. Finally, several rare tumor types originated from specific MEC populations. Lipid-rich tumors arose primarily from the luminal CD133+ cell population, whereas sebaceous-like carcinoma developed primarily from basal and stem cells. Thus, each MEC-enriched population

generated tumors with a broad but distinct spectrum of histological subtypes.

Estrogen receptor (ER) is a standard clinical marker used to guide a patient's course of treatment and predict clinical outcome (27). ER IHC staining was used to determine if any enriched MEC subgroups developed predominantly ER-positive (ER+) tumors, as defined by strong nuclear staining (Figure 2.2L). Surprisingly, all of the enriched MEC populations gave rise to ER+ tumors, and luminal CD133+ cells generated the highest proportion of ER+ tumors (Figure 2.2M). Interestingly, most of the ER+ tumors exhibited either papillary or acinar histology, whereas few squamous tumors stained positive for ER (Figure 2.2N). These observations are consistent with previous reports showing that ER+ status appears to be associated with well-differentiated tumor histologies in PYMT-oncogene driven tumors, and is less prevalent in tumors that were poorly differentiated (28).

Luminal CD133- MECs give rise to exclusively Basal subtype mammary tumors

We next wanted to determine whether MEC populations influenced the molecular classification of the tumors they generate. Tumors derived from each MEC population were classified by hierarchical analysis of microarray gene expression data. This classification method has been used to group tumors from a variety of mouse models into Basal and Luminal subtypes, similar to those observed in breast cancer (3). We have previously demonstrated that lentiviral-mediated expression of PYMT in unsorted MECs generates both Basal and Luminal tumor subtypes, which is in contrast to the development of only Luminal tumor subtypes in MMTV-PYMT transgenic mice (3,8,15). We analyzed between 6 and 11 tumors generated from each enriched MEC population by microarray gene expression profiling and hierarchical clustering. We used an intrinsic 669-gene set consisting of genes differentially expressed in molecularly Basal and Luminal tumors to determine the molecular subtype (3). Gene set expression data were hierarchically clustered with 12 mouse models of breast cancer. Based on this analysis, we found that luminal CD133+, basal, and stem-enriched MECs were able to give rise to both Basal and Luminal tumors (Figure 2.3), with no correlation to tumor histology or ER status (data not shown). In contrast, the luminal CD133- population, which is enriched in luminal progenitors (18), exclusively established tumors of the Basal subtype. To determine the significance of this

observation, we compared the distribution of Basal and Luminal subtypes generated from each cell population with the distribution we observed when unsorted MECs were transduced with the PYMT lentivirus [8]. Only the luminal CD133- cells established tumors with a significantly different distribution of Basal and Luminal subtypes (p=0.017, Supplementary Table 2.1). This finding was surprising, but not unprecedented, since luminal progenitors are proposed to be the cellular origin for Basal-like breast cancer observed in mouse models and patients carrying mutations in BRCA1 (4,10). These data further support luminal progenitors as a cellular origin for Basal-like tumors.

Luminal CD133+ cells give rise to tumors that are significantly less metastatic than other tumor groups

We investigated whether the enriched MEC subgroups expressing PYMT generate tumors with different metastatic capacities. Similar to other PYMT-driven models, tumors generated from each MEC subgroup metastasized to the lungs (Figure 2.4A and Supplementary Figure 2.2A); metastasis to other organs was not detected. The metastatic burden in lungs was quantified by wholemount fluorescent imaging and H&E staining of serial lung sections. Interestingly, although no significant differences in tumor prevalence or progression were observed between the four tumor initiating populations (Figure 2.1D), tumors derived from the luminal CD133+ cell population were significantly less metastatic than all other tumor groups (Figure 2.4B). Furthermore, these cells formed significantly fewer lung tumor foci compared to the other tumor groups (Figure 2.4C); however, there was no difference in the average area per metastatic focus (Figure 2.4D). These data suggest that the cell of origin influences metastatic properties of a tumor.

Metastasis progresses through several stages: cells leave the primary site and enter the bloodstream or lymphatics, survive within the circulatory system, exit the circulatory system, and colonize a secondary site (29). To identify differences in the metastatic process between tumor groups, we quantified circulating tumor cells (CTCs, a measure of tumor cell invasion and intravasation into the bloodstream) and assessed metastatic colony formation following systemic injection of tumor cells (a measure of tumor extravasation from the bloodstream, seeding, and

colonization at secondary sites). CTCs expressing fluorescent ZsGreen were quantified in whole blood from tumor-bearing mice by FACS and normalized to both the volume of collected blood and background fluorescence observed in non-tumor-bearing mice. Whole blood from mice with tumors derived from luminal CD133+ cells had significantly fewer CTCs than the other tumor groups (Figure 2.4E). However, when injected into the tail vein, both luminal CD133+ and CD133- tumor cells were able to colonize and proliferate within the lung regardless of whether the donor tumor was metastatic (Figures 2.4F, Supplementary Figure 2.2B). These data suggest that luminal CD133+ cells give rise to tumors with a limited ability to escape the primary cancer and intravasate into the bloodstream.

Discussion

During transformation, a cell converts from a normal to a malignant state through a process requiring genomic and molecular alterations. A critical step is the activation of an oncogenic pathway, generally through genomic mutation, amplification, or overexpression. In the mammary gland, a transformed cell will originate within either the luminal or basal epithelium, where a spectrum of functionally distinct cell populations reside (30). Thus, tumorigenesis occurs within the unique molecular and cellular background of the cell of origin. How this affects the transformation process is not well understood; however, recent studies have demonstrated that the cellular context of a transforming event can affect characteristics of the tumor (9,10,31).

Based on this understanding, we asked whether PYMT expression within different cellular origins of the mammary gland would affect histopathology, molecular subtype, and metastasis of tumors. In a previous study, we expressed PYMT broadly in mammary epithelium using a lentiviral approach, and compared the tumors with those derived from MMTV-PYMT transgenic mice (8). This comparison revealed significant differences in tumor histology and molecular subtype between the models. Mammary tumors derived from MMTV-PYMT mice exhibited a solid adenocarcinoma phenotype and were molecularly classified within the Luminal subtype (3,15). In contrast, tumors driven by the EF1α-PYMT-ZsGreen lentivirus were histologically and molecularly diverse (8), thus demonstrating the PYMT oncogene has the ability

to generate a spectrum of tumor subtypes when it is broadly expressed in mammary epithelial cells.

In the current study, we investigated whether expression of PYMT within specific cell populations alters the phenotypic outcome of tumors. The data demonstrate that each MEC population has the capacity to give rise to diverse tumor pathologies. However, some MEC populations did exhibit a prevalence of certain tumor types. Most striking was that luminal CD133+ cells gave rise to a higher proportion of tumors with papillary histology, ER+ expression, and limited squamous differentiation. Consistent with their well-differentiated pathology, tumors from luminal CD133+ cells also produced fewer CTCs and metastases. An opposing phenotype was observed in tumors generated by enriched stem cells. Notably, these cells gave rise to tumors that were ER negative (ER-), exhibited squamous differentiation, and produced more CTCs and metastases than luminal CD133+ cells. Thus, luminal CD133+ cells largely gave rise to well-differentiated tumors, whereas tumors derived from the enriched stem cell population were generally poorly-differentiated and metaplastic. These data are consistent with that reported by Keller et al., which showed that transformation of CD10-enriched human basal cells established ER- and metaplastic tumors with features of squamous differentiation (31). They proposed that the cellular precursor to metaplastic breast cancer resides within the basal cell layer, a hypothesis also supported by our study using a mouse mammary model. Taken together, these data demonstrate that tumor histopathology and metastatic potential are, at least partly, influenced by the tumor's cellular origin.

Mouse mammary tumor models can be molecularly classified by microarray gene expression profiling into Basal and Luminal tumor subtypes (3). It has been well established that tumors derived from MMTV-PYMT mice exhibit a Luminal subtype (3,15). In contrast, as we previously reported, targeting PYMT expression to all MEC populations, using a lentiviral approach, establishes both Basal and Luminal subtypes (8). Here we demonstrate that both Luminal and Basal tumor subtypes can arise from enriched luminal CD133+, Stem, and Basal mammary cell populations. However, we also show that transformation of CD133- luminal cells, which are enriched for luminal progenitors (18), generated only Basal tumor subtypes. This

finding was surprising and suggests luminal progenitors preferentially establish Basal rather than Luminal subtypes. However, this finding is consistent with several recent observations that attribute Basal-like breast cancer to a luminal progenitor population. Lim et al. demonstrated that the molecular profile of untransformed luminal progenitors most closely resembles Basal-like breast cancers (4). In addition, transformation of human EpCAM+/CD10-/CD49f+ luminal progenitors derived from reduction mammoplasties established tumors with features similar to Basal-like breast cancer, including reduced ER and greater CK14 expression than tumors derived from differentiated luminal cells (31). Furthermore, targeting BRCA1 loss-of-function to luminal cells in mice generated tumors with Basal-like features that closely resemble those observed in patients carrying the BRCA1 mutation. However, the same loss-of-function in basal cells generated adenomyoepitheliomas (6,10). Taken together, these data support luminal progenitors as a potential cellular origin of Basal-like breast cancer.

Conclusions

We report that differentiated luminal (CD133+), luminal progenitor (CD133-), basal, and stem cell-enriched populations have the capacity to give rise to mammary tumors at equivalent frequency and latency when transformed with PYMT oncogene. However, mammary cell populations can produce tumors with differences in histopathology, molecular classification, and metastatic potential. Basal and stem cell-enriched populations predominantly established poorly differentiated squamous tumors, whereas differentiated luminal cells gave rise to a high proportion of estrogen receptor positive papillary tumors. We also demonstrate that the luminal progenitor-enriched population specifically gives rise to tumors with a Basal cancer molecular subtype. These data suggest a tumor's cellular origin can influence a tumor's phenotypic outcome.

Competing Interests

The authors declare that they have no potential conflicts of interest.

Authors' Contributions

DD participated in study design and performed experiments, analyzed data, and drafted the manuscript. BS participated in study design, experiments, data analysis, and edited the manuscript. HAE performed tail vein injections and lung metastasis quantification. BW participated in study design and coordination, and helped draft and edit the manuscript. All authors read and approved the final manuscript.

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References

1. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98(19):10869-74.

2. Malhotra GK, Zhao X, Band H, Band V. Histological, molecular and functional subtypes of breast cancers. Cancer Biol Ther 2010;10(10):955-60.

3. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 2007;8(5):R76.

4. Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 2009;15(8):907-13.

5. Lim E, Wu D, Pal B, Bouras T, Asselin-Labat ML, Vaillant F, et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 2010;12(2):R21.

6. Hennessy BT, Gonzalez-Angulo AM, Stemke-Hale K, Gilcrease MZ, Krishnamurthy S, Lee JS, et al. Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. Cancer Res 2009;69(10):4116-24.

7. Shi P, Wang M, Zhang Q, Sun J. Lipid-rich carcinoma of the breast. A clinicopathological study of 49 cases. Tumori 2008;94(3):342-6.

8. Smith BA, Shelton DN, Kieffer C, Milash B, Usary J, Perou CM, et al. Targeting the PyMT Oncogene to Diverse Mammary Cell Populations Enhances Tumor Heterogeneity and Generates Rare Breast Cancer Subtypes. Genes Cancer 2012;3(9-10):550-63.

9. Ince TA, Richardson AL, Bell GW, Saitoh M, Godar S, Karnoub AE, et al. Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 2007;12(2):160-70.

10. Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R, et al. BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. Cell Stem Cell 2010;7(3):403-17.

11. Raptis L, Marcellus R, Corbley MJ, Krook A, Whitfield J, Anderson SK, et al. Cellular ras gene activity is required for full neoplastic transformation by polyomavirus. J Virol 1991;65(10):5203-10.

12. Webster MA, Hutchinson JN, Rauh MJ, Muthuswamy SK, Anton M, Tortorice CG, et al. Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. Mol Cell Biol 1998;18(4):2344-59.

13. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318(5853):1108-13.

14. Fromowitz FB, Viola MV, Chao S, Oravez S, Mishriki Y, Finkel G, et al. ras p21 expression in the progression of breast cancer. Hum Pathol 1987;18(12):1268-75.

15. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12(3):954-61.

16. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature 2006;439(7072):84-8.

17. Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ. CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res 2006;8(1):R7.

18. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. J Cell Biol 2007;176(1):19-26.

19. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, et al. Purification and unique properties of mammary epithelial stem cells. Nature 2006;439(7079):993-7.

20. Eyob H, Ekiz HA, Derose YS, Waltz SE, Williams MA, Welm AL. Inhibition of ron kinase blocks conversion of micrometastases to overt metastases by boosting antitumor immunity. Cancer Discov 2013;3(7):751-60.

21. Mikaelian I, Hovick M, Silva KA, Burzenski LM, Shultz LD, Ackert-Bicknell CL, et al. Expression of terminal differentiation proteins defines stages of mouse mammary gland development. Vet Pathol 2006;43(1):36-49.

22. Li Z, Tognon CE, Godinho FJ, Yasaitis L, Hock H, Herschkowitz JI, et al. ETV6-NTRK3 fusion oncogene initiates breast cancer from committed mammary progenitors via activation of AP1 complex. Cancer Cell 2007;12(6):542-58.

23. Sun P, Yuan Y, Li A, Li B, Dai X. Cytokeratin expression during mouse embryonic and early postnatal mammary gland development. Histochem Cell Biol 2010;133(2):213-21.

24. Chakrabarti R, Wei Y, Romano RA, DeCoste C, Kang Y, Sinha S. Elf5 regulates mammary gland stem/progenitor cell fate by influencing notch signaling. Stem Cells 2012;30(7):1496-508.

25. Spike BT, Kelber JA, Booker E, Kalathur M, Rodewald R, Lipianskaya J, et al. CRIPTO/GRP78 Signaling Maintains Fetal and Adult Mammary Stem Cells Ex Vivo. Stem Cell Reports 2014;2(4):427-39.

26. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature 2011;479(7372):189-93.

27. Lapidus RG, Nass SJ, Davidson NE. The loss of estrogen and progesterone receptor gene expression in human breast cancer. J Mammary Gland Biol Neoplasia 1998;3(1):85-94.

28. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol 2003;163(5):2113-26.

29. Gupta GP, Massague J. Cancer metastasis: building a framework. Cell 2006;127(4):679-95.

30. Visvader JE. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes Dev 2009;23(22):2563-77.

31. Keller PJ, Arendt LM, Skibinski A, Logvinenko T, Klebba I, Dong S, et al. Defining the cellular precursors to human breast cancer. Proc Natl Acad Sci U S A 2012;109(8):2772-7.

Figure 2.1. A model to assess the influence of the cell of origin on tumor phenotype. A. Freshly isolated MECs were transduced with an Ef1α-PyMT-ZsGreen lentivirus, FACS sorted into distinct populations, and transplanted into the cleared mammary fat pads of syngeneic mice. B. Transduced MECs were sorted into basal, luminal, and stem cell populations based on the expression of the cell surface markers CD49f and CD24 (right). Luminal cells were further sorted according to CD133 expression into hormone-receptor positive (CD133+) and negative (CD133-) populations (left). The collected populations are indicated by red gates. C. FACS-enriched populations were evaluated for expression of basal K14 (red), luminal K8 (green), and DAPI (blue) by immunofluorescence (scale bar: 20μm). D. Quantification of the cytokeratin profile for each MEC subgroup (n: total number of cells imaged). E. Kaplan-Meier curves of mice receiving orthotopic transplants of distinct MEC subgroups. Mice were sacrificed when tumors reached 2-cm in diameter. (n: number of mice).

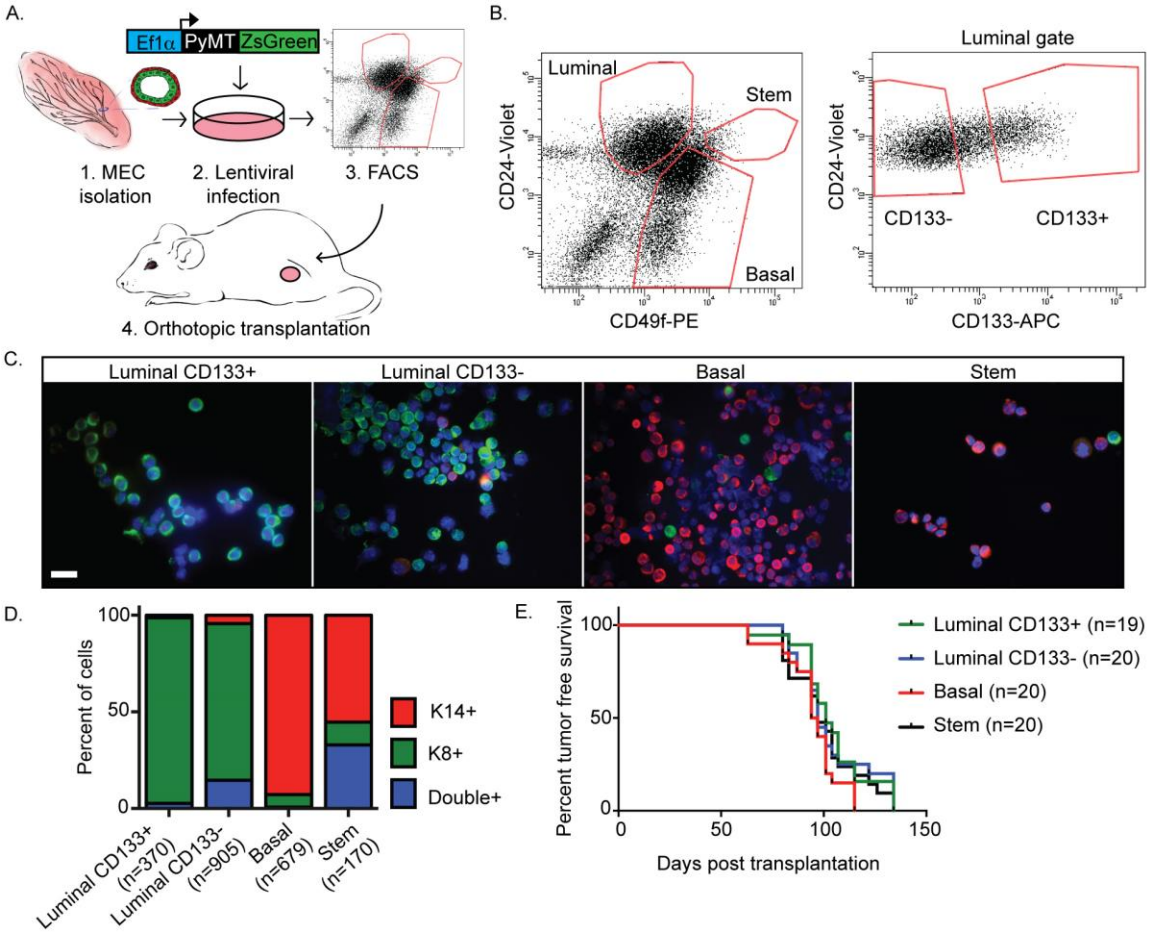

A.
Ef1α PyMT ZsGreen
1. MEC isolation
2. Lentiviral infection
3. FACS
4. Orthotopic transplantation
B.
Luminal
Stem
Basal
CD24-Violet
CD49f-PE
Luminal gate
CD133-
CD133+
CD133-APC
C.
Luminal CD133+
Luminal CD133-
Basal
Stem
D.
Percent of cells
100
50
0
K14+
K8+
Double+
Luminal CD133+ (n=370)
Luminal CD133- (n=905)
Basal (n=679)
Stem (n=170)
E.
Percent tumor free survival
100
50
0
0
50
100
150
Days post transplantation
Luminal CD133+ (n=19)
Luminal CD133- (n=20)
Basal (n=20)
Stem (n=20)

Figure 2.2. Analysis and prevalence of histology in tumors derived from MEC populations. A-F. Representative images of H&E and cytokeratin staining of tumor histologies: acinar (A), papillary (B), solid adenocarcinoma (C), squamous (D), lipid-rich (E), sebaceous-like (F). Immunofluorescence staining was performed for basal K14 and luminal K8 (scale bas: 100μm). Histology area per tumor derived from luminal CD133+ cells (G), luminal CD133- cells (H), basal cells (I), and stem cells (J). K. Average area of histology per MEC group (unpaired t-test, n: number of tumors). L. Representative images of ER staining, including negative (left panel) and positive staining (right panel) (scale bar: 50μm) (n: number of tumors). M. Quantification of ER staining per MEC group (two proportion z-test). N. Quantification of ER staining per histology (two proportion z-test).

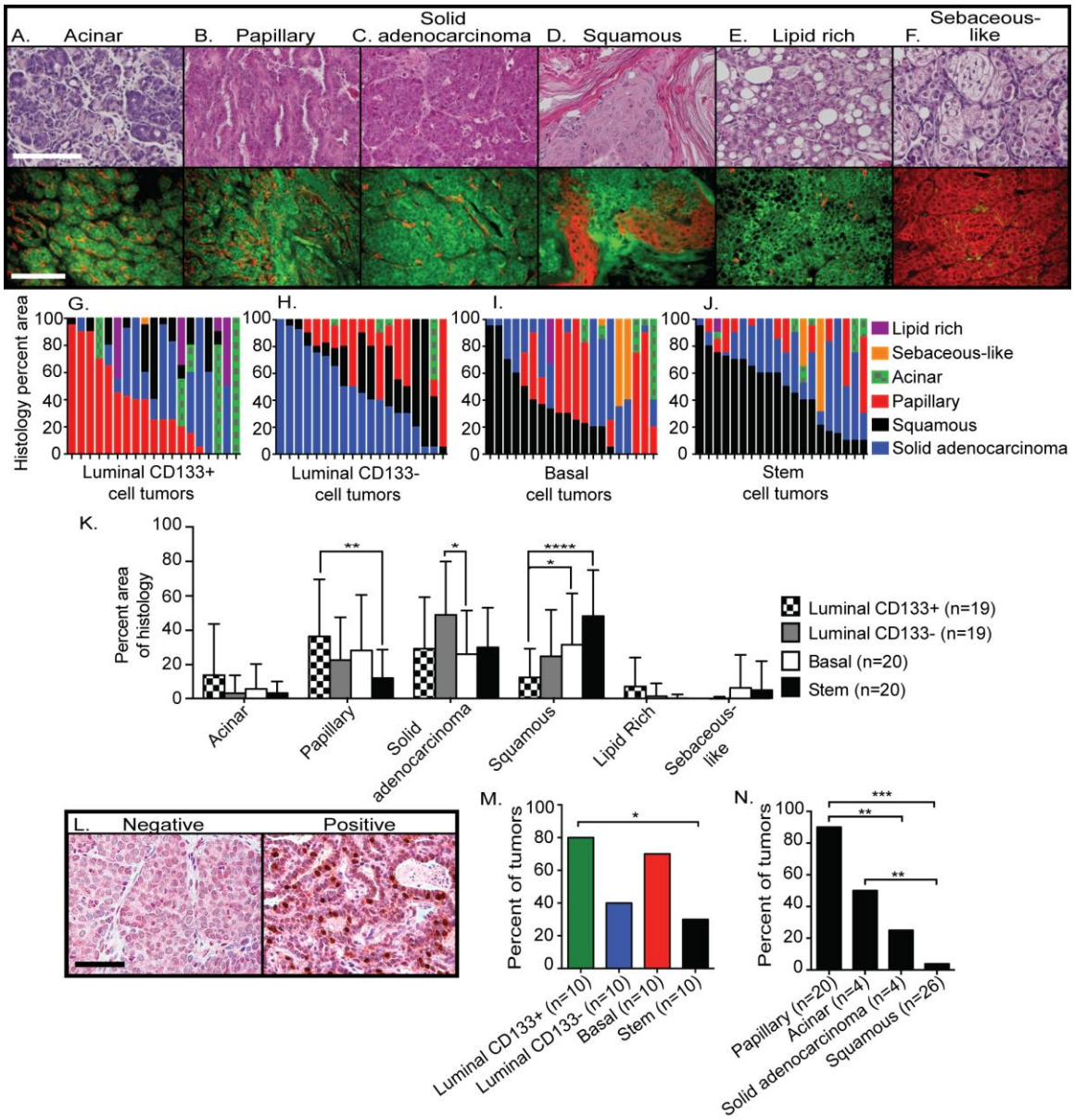

A. Acinar
B. Papillary
C. Solid adenocarcinoma
D. Squamous
E. Lipid rich
F. Sebaceous-like
G.
H.
I.
J.
Histology percent area
Luminal CD133+ cell tumors
Luminal CD133- cell tumors
Basal cell tumors
Stem cell tumors
Lipid rich
Sebaceous-like
Acinar
Papillary
Squamous
Solid adenocarcinoma
K.
Percent area of histology
Luminal CD133+ (n=19)
Luminal CD133- (n=19)
Basal (n=20)
Stem (n=20)
Acinar
Papillary
Solid adenocarcinoma
Squamous
Lipid Rich
Sebaceous-like
L. Negative
Positive
M.
Percent of tumors
Luminal CD133+ (n=10)
Luminal CD133- (n=10)
Basal (n=10)
Stem (n=10)
N.
Percent of tumors
Papillary (n=20)
Acinar (n=4)
Solid adenocarcinoma (n=4)
Squamous (n=26)

Figure 2.3. Tumor microarray gene expression profiling. Tumors were analyzed by microarray gene expression profiling and hierarchically clustered with mouse mammary tumor models using an intrinsic gene set identified by Herschkowitz et al. 2007. Vertical lines indicate individial tumors. Each enriched MEC population is indicated by a different color: green: luminal CD133+; blue: luminal CD133-; red: basal; and, black: stem cells. Mouse mammary tumor models that generate molecularly luminal tumors are shown in dark blue, while those with predominatly basal subtypes are dark red. Normal mouse mammary tissue is colored brown (n: number of tumors).

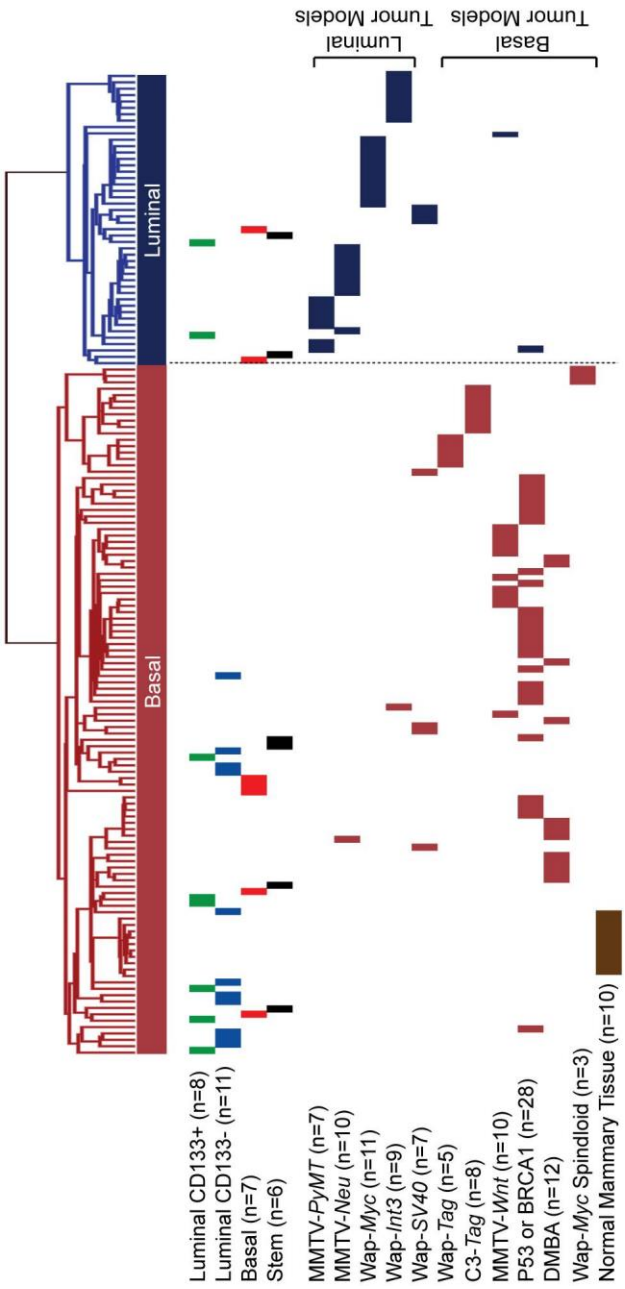
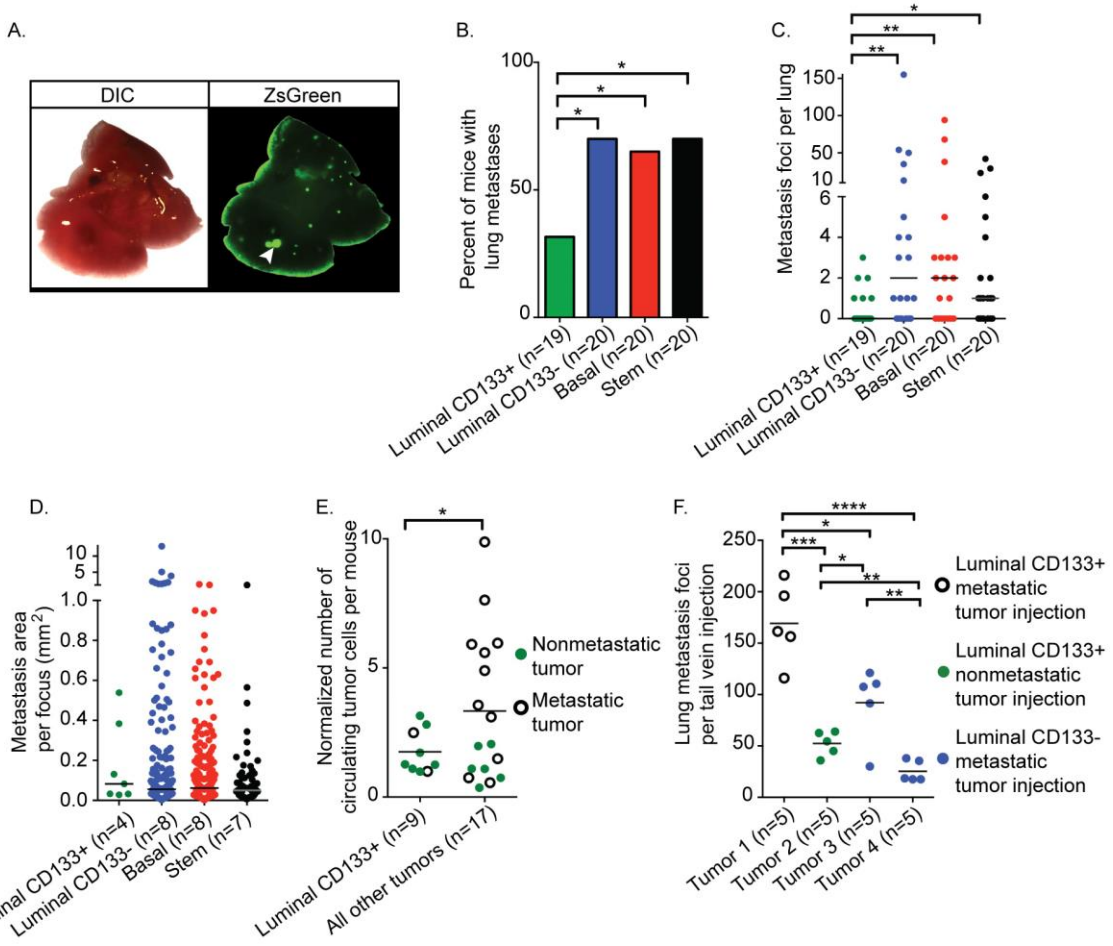
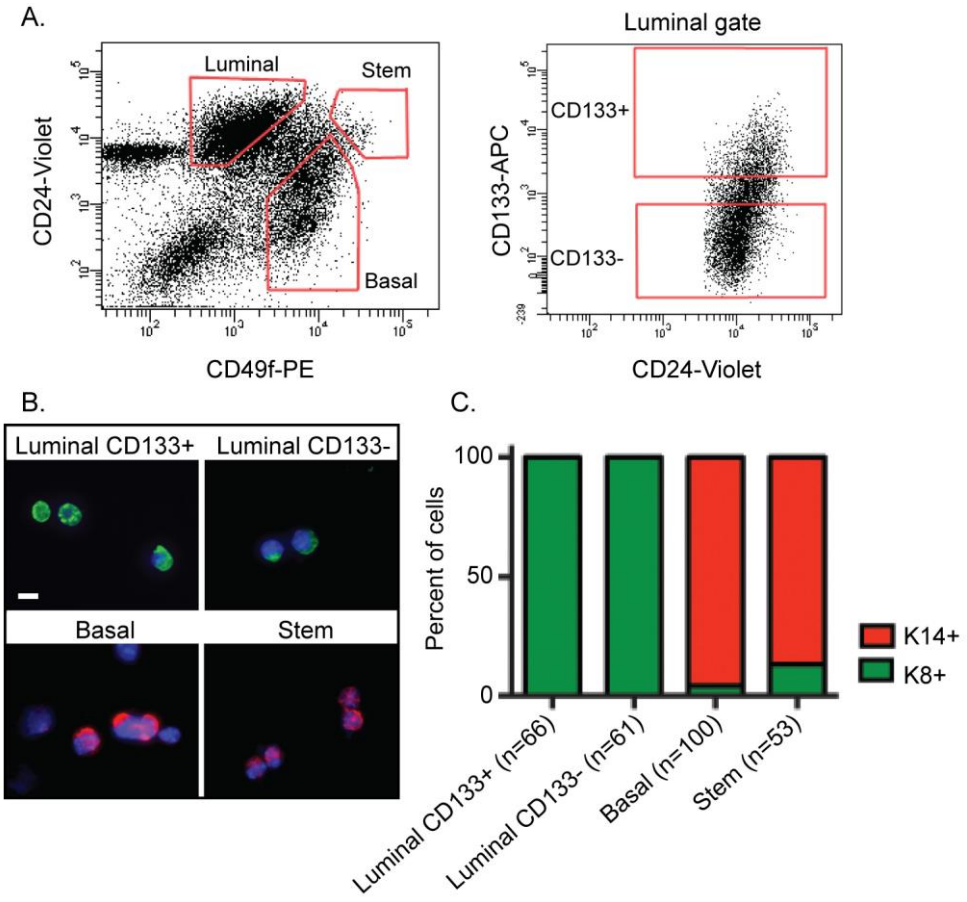

Luminal
Basal
Luminal Tumor Models
Basal Tumor Models
Luminal CD133+ (n=8)
Luminal CD133- (n=11)
Basal (n=7)
Stem (n=6)
MMTV-PyMT (n=7)
MMTV-Neu (n=10)
Wap-Myc (n=11)
Wap-Int3 (n=9)
Wap-SV40 (n=7)
Wap-Tag (n=5)
C3-Tag (n=8)
MMTV-Wnt (n=10)
P53 or BRCA1 (n=28)
DMBA (n=12)
Wap-Myc Spindloid (n=3)
Normal Mammary Tissue (n=10)

Figure 2.4. Metastatic profiles of tumors generated from enriched MEC populations. A. Representative DIC (left panel) and fluorescent (right) images of the same metastatic lung. Arrowhead indicated the same metastatic site. Black and white fluorescent images were false colored green in ImageJ and overlaid on a black background. ZsGreen expression is shown in green. B. Percent of mice with lung metastases per tumor group. Luminal CD133+ cell tumors were less metastatic than the other tumor groups (two proportion z-test; n: number of mice). C. Number of metastatic lung foci per tumor group. Luminal CD133+ cell tumors generated fewer metastatic foci than the other tumor groups (Mann-Whitney test, medians shown). D. Quantification of metastasis area per unique metastatic site in serial lung sections. No difference in tumor metastasis size was detected between the tumor groups (n: number of mice). D. Normalized number of circulating tumor cells in mice with luminal CD133+ tumors compared to all other tumor groups. ZsGreen signal in whole blood isolated from tumor-bearing mice was analyzed by FACS and normalized to no-tumor control signal. Luminal CD133+ tumor-bearing mice had fewer tumor circulating cells. Mice with nonmetastatic tumors are represented by green, and those with metastatic tumors are represented by black (unpaired t-test, means shown; n: number of mice). F. Quantification of the number of lung tumor foci per tail vein injection of metastatic luminal CD133+ tumor cells (Tumor 1), nonmetastatic luminal CD133+ tumor cells (Tumor 2), or metastatic luminal CD133- tumor cells (Tumors 3 and 4) (unpaired t-test, means shown; n: number of mice).


A.
DIC
ZsGreen
B.
Percent of mice with lung metastases
100
50
0
Luminal CD133+ (n=19)
Luminal CD133- (n=20)
Basal (n=20)
Stem (n=20)
C.
Metastasis foci per lung
150
100
50
10
6
4
2
0
D.
Metastasis area per focus (mm2)
10
5
1.0
0.8
0.6
0.4
0.2
0.0
Luminal CD133+ (n=4)
Luminal CD133- (n=8)
Basal (n=8)
Stem (n=7)
E.
Normalized number of circulating tumor cells per mouse
10
5
0
Luminal CD133+ (n=9)
All other tumors (n=17)
Nonmetastatic tumor
Metastatic tumor
F.
Lung metastasis foci per tail vein injection
250
200
150
100
50
0
Tumor 1 (n=5)
Tumor 2 (n=5)
Tumor 3 (n=5)
Tumor 4 (n=5)
Luminal CD133+ metastatic tumor injection
Luminal CD133+ nonmetastatic tumor injection
Luminal CD133- metastatic tumor injection

Supplementary Figure 2.1. Robust enrichment of specific MEC populations. A. Transduced MECs were sorted into basal, luminal, and stem cell populations based on the expression of cell surface markers CD49f and CD24 (right). Luminal cells were further sorted according to CD133 expression into hormone receptor positive (CD133+) and negative (CD133-) populations (left). Collected populations are indicated by red gates. B. FACS-enriched MEC populations were stained for basal K14 (red), luminal K8 (green), and DAPI (blue) (scale bar: 10μm). C. Quantification of the cytokeratin profile for each MEC subgroup (n: total number of cells imaged).


A.
Luminal
Stem
Basal
CD24-Violet
CD49f-PE
Luminal gate
CD133+
CD133-
CD133-APC
CD24-Violet
B.
Luminal CD133+
Luminal CD133-
Basal
Stem
C.
Percent of cells
100
50
0
K14+
K8+
Luminal CD133+ (n=66)
Luminal CD133- (n=61)
Basal (n=100)
Stem (n=53)

Supplementary Figure 2.2. Enriched MEC populations metastasize to lung. A. Representative image of H&E staining of a metastatic lung section. Arrow indicates metastasis (scale bar: 1mm). B. Representative fluorescent lung images from mice that received tail vein injections of metastatic luminal CD133+ tumor cells (Tumor 1), nonmetastatic luminal CD133+ tumor cells (Tumor 2), or metastatic luminal CD133- tumor cells (Tumors 3 and 4). Lungs were examined three weeks after the tail vein injection. Fluorescent black and white lung images were false colored green in ImageJ and overlaid on a black background.

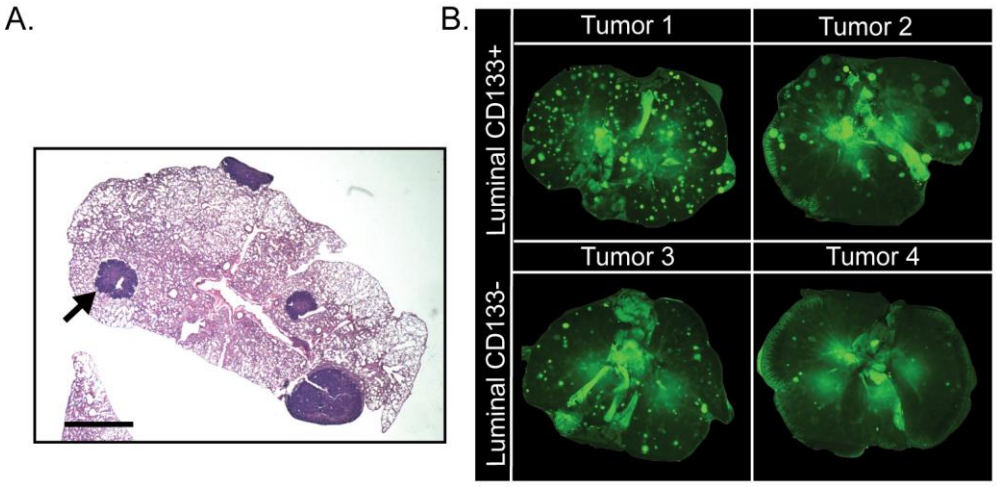

A.
B.
Tumor 1
Tumor 2
Tumor 3
Tumor 4
Luminal CD133+
Luminal CD133-

Table 2.1. Description and summary of tumor histologies.

Tumor Histology	**Overall Cellular Organization**	**Histology Description**	**Cytokeratin Staining**
Acinar	Well differentiated	Acini with duct-like bilayered epithelial cells	Maintenance of ductal morphology with K8 cells surrounding clear lumens and K14 cells adjacent to the stroma
Papillary	Well differentiated	Epithelial cell sheets, generally 2-3 layers thick, surrounded by stroma	Maintenance of luminal and basal cell organization with K14 positive cells adjacent to the stroma
Solid Adenocarcinoma	Poorly differentiated	Multilayered epithelial cells grouped in large units (>100 um diameter) surrounded by 1-2 layers of stromal cells	Primarily K8 positive with sparse K14 positive cells near stromal regions
Squamous	Poorly differentiated	Adenocarcinoma with clusters of keratin pearls and sheets of squamous epithelia	Large solid areas of either K8 or K14 positive cells. K14 cells are localized near keratinized regions
Lipid Rich	Poorly differentiated	Heavily vacuolated epithelial cells	Primarily K8 positive
Sebaceous-like	Poorly differentiated	Epithelial cells with enlarged nuclei and foamy cytoplasms	Primarily K14 positive

Supplementary Table 2.1. Comparison of the distribution of Basal and Luminal tumor subtypes generated from each cell population with the distribution obtained from unsorted MECs [8], using Fisher's exact test.

Transformed population	**Basal subtype**	**Luminal subtype**	**P-value**
Luminal CD133+	6	2	0.6757
Luminal CD133-	11	0	0.0172
Basal	5	2	1.0000
Stem	4	2	1.0000
Unsorted [8]	14	9	

CHAPTER III

TARGETING GENE EXPRESSION TO SPECIFIC MOUSE MAMMARY EPITHELIAL CELL POPULATIONS USING LENTIVIRAL INFECTION AND CRE-MEDIATED RECOMBINATION

Introduction

Breast cancer is a heterogeneous disease, which consists of diverse molecular and histological subtypes that vary in their clinical outcomes (1,2). Heterogeneity in breast cancer may be influenced by both the intrinsic factors of cancer cells, and extrinsic contributions of the tumor's microenvironment and systemic hormones. Intrinsic factors include differences in molecular and genetic properties of the cell type that gave rise to the tumor, and genetic alterations that were acquired during tumor progression (3). While genetic alterations in cancer cells are known to be important effectors of tumor phenotype, recent studies are providing evidence that inherent factors of the cell from which the cancer originated can also influence tumor phenotype.

Breast cancer can be divided into subtypes that are classified by the expression of specific molecular markers, many of which are associated with distinct mammary epithelial cells (MECs). For example, luminal breast cancers and normal luminal cells upregulate similar cytokeratins and hormone receptor pathways. The gene expression profiles of Basal-like breast cancers resemble those of normal luminal progenitors and basal populations. However, claudin-low tumors express genes associated with stem cells (2,4-6). While these findings are correlative, they do suggest that some breast cancer subtypes may originate from distinct MEC populations.

Experimental evidence further supports the hypothesis that distinct MEC populations give rise to specific breast cancer types. For example, Ince et al. generated unique tumor types from

two distinct mammary epithelial cell populations, demonstrating the transformed cell type influences cancer phenotype (7). Furthermore, several reports show that luminal progenitors are the origins of BRCA1-mutant Basal-like cancer (6) (8). Although informative, these studies were performed using *in vitro* approaches or a limited number of transgenic mouse mammary tumor models. As a result, it is still unknown whether other MEC populations give rise to specific tumor types. Therefore, more cell type-specific models are needed to identify the tumor cells of origin of other breast cancer subtypes.

Many current transgenic mouse mammary tumor models utilize hormone-activated promoters to drive oncogene expression in the mammary epithelium. These include the mouse mammary tumor virus long terminal repeat (MMTV) and whey acidic protein (WAP) promoters (9-11). MMTV and WAP transgenic mice have been used to successfully generate mammary tissue-specific tumors, but these models fail to recapitulate the full molecular and histological tumor diversity observed in breast cancer (12,13). This may be due to the fact that WAP and MMTV promoters are primarily active in subsets of hormone-responsive luminal cells (13,14). However, the mammary gland epithelium consists of multiple cell types, including stem, basal, and luminal populations(15), and the MMTV and WAP promoters are unable to drive gene expression in all MEC types. Unfortunately, generating transgenic mouse mammary tumor models that can express a wide variety of oncogenes in specific MEC populations is difficult and not cost effective.

As an alternative to generating transgenic mouse models, Welm et al. demonstrated that primary MECs can be transduced with a lentivirus expressing the enhanced green fluorescent protein (EGFP) under control of a ubiquitous elongation factor-1 alpha (EF1α) promoter. Subsequent transplantation of infected cells into the cleared mammary fat pads of recipient mice resulted in regeneration of functional mammary glands that expressed EGFP in all MEC populations, including luminal, basal, and stem cells (16). The benefit of this lentiviral infection and transplantation approach is that the lentivirus can be easily modified to express a wide variety of genes in the mammary epithelium; however, this model is limited in its ability to drive gene expression in a cell-specific manner.

Therefore, the aim of this chapter is to describe a protocol that combines lentiviral infection and transplantation techniques with Cre-mediated recombination to activate any gene expression in specific mouse mammary epithelial cell populations. In this approach, lentiviral constructs were designed to express a gene, oncogene, or fluorescent marker after Cre-mediated recombination. Next, MECs isolated from mice that express Cre under the control of lineage-specific promoters were infected with the lentivirus. Transduced cells were then used for orthotopic transplantation for lineage tracing and tumor generation. Unlike the previous lentiviral and transgenic mouse mammary tumor models, this novel approach can be used to test the tumor cell of origin hypothesis by targeting oncogene expression to specific MEC populations.

Results

Lentiviral plasmid design and characterization

Transgenic mouse models have extensively utilized Cre-mediated recombination at LoxP-flanked DNA regions to activate or suppress gene expression (17). As an alternative to generating a transgenic mouse model, we developed Cre-activated lentiviral constructs to target gene expression to cells with Cre recombinase activity. These constructs consist of a ubiquitous EF1α promoter (16) upstream of a LoxP-flanked translational stop cassette, which contains a histone H2B-fused enhanced green fluorescent protein (H2B-EGFP). To demonstrate that Cre-expressing cells can be labeled using the lentiviral construct, a fluorescent Tomato reporter was inserted downstream of the stop cassette (Figure 3.1A). To transform Cre-expressing cells, the polyoma middle T antigen (PYMT) oncogene was inserted downstream of the stop cassette (Figure 3.1B). Thus, the lentiviral constructs were designed to express Tomato or PYMT after Cre-mediated recombination.

The lentiviral plasmid encoding Tomato downstream of the LoxP-flanked stop cassette (henceforth termed LoxP-Tomato) was used as a reporter for Cre activity. To validate construct design, 293T cells were transfected with the LoxP-Tomato plasmid with or without a Cre-expression plasmid (18). Fluorescence activated cell sorting (FACS) of plasmid-transfected cells demonstrated robust EGFP expression in LoxP-Tomato transfected cells, and Tomato was

expressed only after Cre-mediated LoxP recombination (Figure 3.1C). Because the translational stop cassette efficiently inhibited Tomato expression in the absence of Cre, the LoxP-Tomato lentivirus can be used to differentially label Cre-expressing cell populations.

The lentiviral construct encoding PYMT downstream of the LoxP-flanked translational stop cassette (henceforth termed LoxP-PYMT) was also validated by transfecting 293T cells with or without a Cre-expression plasmid (18). Western blot analysis demonstrates that HA-tagged PYMT protein was detected only in cells that expressed Cre (Figure 3.1D). Thus, the LoxP-PYMT lentivrus can be used to transform Cre-expressing cells.

Using the described letiviruses, specific MEC populations could be labeled with Tomato or transformed with the PYMT oncogene by infecting cells isolated from transgenic mice that express Cre under the control of population-specific promoters (several mammary cell-specific Cre lines are summarized in Supplementary Figure 3.1). Then, transduced cells could be grown in a three-dimensional matrix or transplanted into the cleared mammary fat pads of syngeneic mice for analysis (Figure 3.1E).

Targeting gene expression to luminal MECs

Because MMTV-Cre transgenic mice efficiently express Cre in the mammary gland epithelium (11), MECs isolated from these mice were used to characterize the lentiviral system *in vitro* and *in vivo*. Prior to infecting MECs from MMTV-Cre mice, the cell population that expressed Cre was validated. MMTV-Cre transgenic mice are reported to express Cre in the luminal cell population (11,13). However, several studies show that the MMTV promoter is also active in other MEC lineages (19,20). These disparate findings may be attributed to differences in genetic backgrounds of transgenic mice or variable transgene insertion sites.

To identify Cre positive cells, MMTV-Cre mice were crossed with a Cre reporter line. Cre reporter mice express membrane-targeted Tomato (mT) prior to Cre-mediated recombination, and membrane-targeted green fluorescent protein (mG) after recombination (mTmG) (21). *In vitro* three-dimensional (3D) analyses of cells isolated from MMTV-Cre;mTmG mice demonstrated GFP expression in luminal keratin 8 (K8)-positive cells, and not basal keratin 14 (K14)-positive

cells (Figure 3.2A and B). Similar results were observed after imaging whole mammary duct fragments isolated from MMTV-Cre;mTmG mice (Figure 3.2C and D). Based on these data, we concluded that our mice express Cre in luminal MECs.

Next, to show that our lentiviral and transplantation system can be used to target gene expression to specific cell types, MMTV-Cre MECs were infected with the LoxP-Tomato lentivirus. Infected MECs were cultured in 3D and cytokeratin staining demonstrated Tomato expression in luminal K8 positive cells (Figure 3.3A), and not basal K14 positive cells (Figure 3.3B). Furthermore, similar results were observed after infected MMTV-Cre MECs were transplanted into the cleared mammary fat pats of syngeneic background mice: 93% of transplants gave rise to fluorescent outgrowths, and Tomato was expressed only in luminal K8 positive cells (Figures 3.3C-F). Therefore, the described lentiviral system can target gene expression to MECs in a population-specific manner, and cells can be analyzed using *in vitro* and *in vivo* applications.

It should be noted, however, that most of the LoxP-Tomato infected MEC outgrowths were singly labeled with Tomato, and only one outgrowth contained EGFP- and Tomato-positive cells (Figure 3.3C). This result may be due to the fact that only about 25-30% of cells get infected prior to transplantation (data not shown). It is possible that luminal cells may be more readily transduced than other MEC populations. As a result, most of the infected MMTV-Cre outgrowths were Tomato-positive.

As a control, MECs isolated from FVB/NJ mice were infected with LoxP-Tomato and transplanted. This was done to test whether Tomato is expressed in the absence of Cre. Seven out of eight transplants generated fluorescent outgrowths, and all were singly labeled with EGFP (Supplementary Figure 3.1A). Confirming, that gene expression downstream of the LoxP-flanked stop cassette is inhibited in the absence of Cre. Furthermore, keratin staining of mammary duct fragments demonstrated that both luminal and basal populations could be infected (Supplementary Figures 3.2B and C). Additional studies need to be performed to determine why most of the MMTV-Cre outgrowths were singly labeled with Tomato, even though both basal and luminal populations were labeled in several control transplants. We did observe that fluorescent

outgrowths consisted of both infected and uninfected cells; therefore, the singly labeled MMTV-Cre outgrowths are likely originating from a mixture of infected and uninfected cells.

Targeting gene expression to basal and stem MECs

In addition to luminal cells, the mammary epithelium is composed of basal and stem cell populations (15). Therefore, our lentiviral system could also be used to target gene expression to these populations using MECs isolated from mice that express Cre under the control of basal and stem cell promoters. These include K14 and leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) promoters, which have been shown to activate Cre expression in mammary gland basal and stem cells, respectively (8). Furthermore, basal cells upregulate smooth muscle proteins as they differentiate into contractile myoepithelial cells (22). Therefore, smooth muscle 22-alpha (SM22α) and smooth muscle myosin heavy chain (SMMHC) promoters may drive Cre expression in more differentiated basal myoepithelial cells (15,23,24).

To test whether our lentiviral system can also be used to target gene expression to mammary basal and stem cells, MECs isolated from LGR5-CreER, K14-CreER, SMMHC-Cre, and SM22α-Cre mice were infected with LoxP-Tomato and cultured in 3D. As expected, Tomato reporter expression was detected in the basal and not luminal cell layer of K14-CreER, SMMHC-Cre, and SM22α-Cre 3D mammary cell cysts (Figures 3.4A-C). In LGR5-CreER mammary cell 3D cultures, Tomato localized to stem cells that sparsely populate the basal layer (Figure 3.4D) (25). These *in vitro* data demonstrate that our system can be used to deliver gene expression to multiple distinct mammary epithelial cell populations, including basal and stem cells.

Transformation of luminal MECs

Next, we generated the LoxP-PYMT letivirus, to target oncogene expression to specific MEC populations. This lentivirus was developed to test whether specific MEC populations influence breast cancer phenotype. The PYMT oncogene was selected as the driver of cellular transformation because it activates similar pathways that are perturbed in breast cancer, including RAS and AKT (26,27).

Initially, to test whether luminal cells could be transformed using our lentiviral system, MECs isolated from MMTV-Cre mice were infected with LoxP-PYMT and transplanted. As a negative control, MECs isolated from FVB/NJ mice were also infected with LoxP-PYMT and transplanted. In both experiments, $1X10^5$ transduced cells were transplanted per mouse. All MMTV-Cre MEC transplants gave rise to tumors with an average latency of 132 days. Surprisingly, we observed that 43% of FVB/NJ transplants also gave rise to tumors with an average latency of 142 days (Figure 3.5A). These tumors grew in the absence of any Cre recombinase activity (data not shown).

To identify the mechanism driving tumor formation in the control mice, we sequenced the lentiviral DNA integrated into the genomes of transformed cells. Interestingly, all tested tumors exhibited homologous recombination at LoxP DNA regions (Figures 3.5B). The fact that tumors arose due to LoxP recombination in the absence of Cre in control transplants was surprising, but not unprecedented since examples of homologous recombination in transfected cells have been described. Rubnitz et al. demonstrated that homologous DNA plasmid sequences as little as 14 bases could recombine in transfected cells (28). Therefore, it is probable that some homologous recombination occurred at plasmid LoxP sites in cells that were transfected during the production of lentivirus, resulting in recombined lentiviral particles.

As described in Figures 3.1, 3.3, and Supplementary Figure 3.2, inappropriate Tomato or PyMT expression was not detected during plasmid and lentivirus characterization. Therefore, homologous recombination in the absence of Cre was likely a rare event. This rare inappropriate recombination was detected only after potent PYMT oncogene expression. Fewer tumors with longer latencies from FVB/NJ cell transplants further suggest that the frequency of homologous recombination in the absence of Cre was rare.

Unfortunately, due to this inappropriate recombination, we were unable to distinguish tumors that arose from cells expressing Cre and cells that did not express Cre. To resolve this, MECs isolated from MMTV-Cre;mTmG mice were used for LoxP-PYMT lentiviral infection and transplantation. As a result, tumors that arose from Cre recombination could be labeled with GFP, and tumors that arose from non-Cre mediated homologous recombination would be Tomato-

positive. As a control, MECs isolated from mTmG mice were also infected with LoxP-PYMT and transplanted. Furthermore, to reduce the number of tumors arising from non-Cre-mediated recombination, $2.5x10^4$ infected cells were transplanted per mouse. In fact, the lower number of transplanted cells decreased the number of tumors in control mice to 20%. The lower number of transplanted cells did not change the latencies of tumors generated from MMTV-Cre;mTmG mouse MECs (Figure 3.5A).

The number of GFP and Tomato labeled tumors derived from mTmG and MMTV-Cre;mTmG MECs was determined using FACS. We identified tumors that consisted of only Tomato- or GFP-positive cells, and tumors that consisted of a mixture of GFP- and Tomato-positive cells (Figure 3.5C). As expected, all tumors derived from mTmG mouse MECs were Tomato-positive, demonstrating that these tumors arose from cells that do not express Cre (Figure 3.5D). Conversely, MMTV-Cre;mTmG mouse MECs gave rise to tumors consisting exclusively of GFP- or Tomato-positive cells, and tumors consisting of both Tomato- and GFP-positive cells. These results show that MMTV-Cre;mTmG MECs generated tumors derived from Cre expressing cells, and cells that do not express Cre. Although we were unable to inhibit aberrant lentivirus recombination, using an mTmG Cre reporter system allowed us to identify tumors that arose from the expected MEC population.

The ultimate goal of our mammary tumor model is to investigate whether different MEC populations contribute to the phenotypes observed in breast cancer. Tumor histology is one way to classify cancer subtype. Previous studies show that MMTV-PYMT transgenic mice typically develop solid adenocarcinomas (9). It is not clear, however, whether this phenotype is specific to PYMT-driven pathogenesis, or if the cell of origin targeted by the MMTV promoter preferentially gives rise to adenocarcinomas. In a recent study, Smith et al. reported that targeting expression of PYMT in a variety of MEC populations using a lentiviral approach resulted in diverse histopathology, dissimilar to what is observed in the MMTV-PYMT model. This study demonstrated that PYMT-driven pathogenesis does not inherently establish solid adenocarcinomas (29). To determine whether MMTV-expressing luminal cells preferentially give rise to solid adenocarcinomas, tumors derived from MMTV-Cre;mTmG and mTmG MECs were

examined by hematoxylin and eosing (H&E) staining. Most of the GFP-positive tumors derived from MMTV-Cre;mTmG MECs were solid adenocarcinomas with papillary and squamous regions (Figure 3.5J), while Tomato/GFP mixed tumors appeared to be much more heterogeneous. Overall, diverse histologies were identified, including solid adenocarcinoma, papillary, squamous, lipid-rich, and sebaceous-like (Figures 3.5E-I). Furthermore, several single tumor masses consisted of multiple histologies. Although these data suggest that solid adenocarcinomas may preferentially arise from luminal cells, more tumors need to be examined to ascertain the significance of these findings.

Transformation of other MEC populations

Next, PYMT expression was targeted to other MEC populations by infecting cells isolated from K14-CreER;mTmG, SM22a-Cre;mTmG, SMMHC-Cre;mTmG, K8-CreER;mTmG, and LGR5-CreER;mTmG mice with LoxP-PYMT. Following infection, $2.5x10^4$ cells were transplanted into the cleared mammary fat pads of syngeneic background mice to generate tumors. Tamoxifen was administered at two different time points to mice receiving transplants of CreER cells. One cohort of transplants received tamoxifen at 1 week post transplantation (WK1), the second cohort was dosed at 4 weeks (WK4). Tamoxifen administration at two different time points was performed with the aim of activating PYMT expression in either progenitor, or more differentiated MEC populations.

Tumor latencies and numbers of mixed, Tomato-positive, or GFP-positive tumors are summarized in Table 3.1. Interestingly, tumors generated from LGR5-CreER;mTmG, K14-CreER;mTmG, and SM22a-Cre;mTmG were primarily Tomato-positive; however, the frequency of these tumors was much higher than mTmG MEC-derived tumors. Further experiments need to be performed to determine whether some cell populations are resistant to transformation. In addition, histological and molecular analyses of the tumors need to be performed.

Discussion and Future Directions

This chapter describes a lentiviral infection and transplantation model that can be used to target gene expression to specific MEC populations. Our goal was to determine whether a lineage-targeted lentiviral approach may serve as a more accessible and versatile method than transgenic mouse models for studying genes involved in mammary gland development and pathogenesis.

To validate our system, fluorescent Tomato was successfully targeted to the luminal lineage using the LoxP-Tomato lentivirus and MECs isolated from MMTV-Cre mice. Interestingly, most of the LoxP-Tomato infected and transplanted MMTV-Cre cells gave rise to singly labeled outgrowths. As expected, the outgrowths expressed Tomato in the luminal cell population, but EGFP expression was lost in most outgrowths. This result may be due to several reasons. For example, basal cells could be more resistant to lentiviral infection, or more luminal cells survived the infection and transplantation procedures. Fluorescent imaging of outgrowths generated from MECs that express Cre in basal populations may help answer this question. Also, FACS can be used to enrich the number of infected cells prior to transplantation, which may enhance the generation of double-labeled outgrowths. Despite these findings, which may not be important for *in vitro* and tumor studies, our data demonstrate that genes can be targeted to specific MEC populations using the described lentiviral system.

Furthermore, we demonstrate that our lentiviral model can be used to transform specific MEC populations; however, tumors also arose due to inappropriately recombined lentivirus. Therefore, a Cre reporter is required to identify tumors arising from Cre expressing cells. These findings should be used as a caution for other investigators that utilize similar transduction techniques to generate tumors. For example, analogous to our lentiviral system, Marumoto et al., developed a series of Cre-loxP–controlled lentiviral vectors that express oncogenes downstream of a LoxP-flanked stop cassette. These lentiviruses were then used to generate a gliablastoma multiforme mouse model (30). In a later publication, Friedmann-Morvinski et al. used the same lentiviral system to demonstrate that neurons and astrocytes can de-differentiate and give rise to gliomas (31). Although the published findings may be valid, neither study reported a control

experiment in which mouse cells that do not express Cre were infected to see if tumors could be generated in the absence of Cre. Furthermore, an internal reporter system, such as mTmG, was not used to label tumors arising from cells that express Cre. Therefore, to avoid any potential aberrant tumor growth, it would be important to investigate whether a lentiviral oncogene delivery system is tightly regulated in all tissues prior to tumor analysis.

Regardless of the caveats associated with our oncogenic lentiviral system, it can still be used for broad applications to investigate breast cancer pathogenesis. Several recent studies have demonstrated that the luminal progenitor population is likely the cell of origin for BRCA1-mutant breast cancer (6,8,32). Other mutations frequently found in breast cancer may also preferentially lead to tumor development in distinct MEC populations. One benefit of our system is that our lentiviral constructs can be easily modified to express different oncogenic factors. For example, future studies can utilize our methodology to express activated Neu (33) in specific MEC populations to investigate whether HER2-postive breast cancers preferentially arise from distinct cell types.

Finally, one way to improve the lentiviral model described in this chapter is by delivering the lentivirus directly to the mammary gland epithelium through nipple injection. The procedure of liquid injection into the nipples of mice has been described previously (34). Injecting lentivirus into the mammary gland through the nipple would eliminate the need for transplantation and reduce any potential side effects due to inflammation. Also, this approach will decrease the number of mice used, and lower the need for inbreeding to ensure that transplant rejection does not occur. We have had some success in delivering dye as well as lentivirus to the mammary epithelium by nipple injection; unfortunately, our results were inconsistent. Optimization of the injection technique may be improved through more experiments.

Methods

Mice

All studies using mice were carried out in strict accordance with University of Utah IACUC guidelines. Surgeries were performed under isofluorane anesthesia, and all efforts were made to minimize animal suffering and prevent infection. Mice used for tumor surgeries were monitored

and euthanized once tumors reached 2cm in diameter. Mice were euthanized using carbon dioxide, followed by cervical dislocation.

FVB/NJ (stock number 001800), LGR5-CreER (stock number 008875), K14-CreER (stock number 005107), SMMHC-Cre (007742), SM22α-Cre (stock number 004746), and mTmG (stock number 007576) mice were obtained from The Jakson Laboratory. MMTV-Cre mice were a generous gift form Tiffany Seagroves, University of Tennessee. K8-CreER mice were a generous gift from Li Xin, Baylor College of Medicine. All mice were backbred to FVB/NJ mice at least ten generations prior to performing transplantation experiments.

Plasmid and lentivirus generation

LoxP-Tomato and LoxP-PYMT lentiviral plasmids were generated over several cloning steps. First, Brittni Smith constructed a plasmid containing the EF1a-LoxP-H2B-EGFP-stop-LoxP sequence. To create the plasmid, the IRES and ZsGreen were removed from the previously described HIV-ZsGreen plasmid (plasmid # 18121, Addgene). An oligo was generated containing the LoxP sites along with a multiple cloning site (top oligo sequence: CCGGGAATTCATAACTTCGTATAATGTATGCTATACGAAGTTATGGATCCATGCAT CCCGGGCCTAGGATAACTTCGTATAATGTATGCTATACGAAGTTATTCTAGACCGG, bottom oligo sequence: CCGGTCTAGAATAACTTCGTATAGCATACATTATACGAAGTTATCCTAGGCC CGGGATGCATGGATCCATAACTTCGTATAGCATACATTATACGAAGTTATGAATTCCCGG). The oligo was inserted using enzyme sites EcoR1 and Xba1 into the above backbone. Then, H2B-EGFP was PCR amplified from a previously described plasmid (plasmid #11680, Addgene) and ligated into the backbone using cut sites BamHI and NsiI. LoxP-Tomato and LoxP-PYMT lentiviral vector cloning was completed by PCR amplifying Tomato and PYMT from previously described plasmids (pHIV-dTomato [Addgene plasmid # 21374], EF1α-PYMT (29)). Then, the amplicons were inserted into the EF1α-LoxP-H2B-EGFP-LoxP backbone using NsiI and KpnI restriction sites. PYMT was amplified using a HA-tagged reverse primer. The LoxP-Tomato and LoxP-PYMT lentiviral vectors were used to produce high titer lentivirus as described previously (29).

Lentiviral plasmid validation

The LoxP-Tomato and LoxP-PYMT lentiviral plasmids were tested by transfecting 293T cells with or without a Cre expression plasmid (pBS513 EF1alpha-cre, plasmid # 11918, Addgene) (18). First, 293T cells were grown to 90% confluence in a 10cm culture plate at 37^{o}C, using 293T culture media (DMEM + 10% fetal bovine serum [FBS]). Transfections were performed after changing the 293T media and adding 1mL of Opti-Mem media (Invitrogen) with 30μg polyethylenimine (Sigma), and 2.5μg of each plasmid DNA, per plate. The cells were allowed to incubate overnight at 37^{o}C. The following day, the transfection media was removed and cells were cultured for 48 hours in 293T culture media prior to analysis. Then, cells transfected with the LoxP-Tomato lentiviral plasmid were analyzed by FACS using a FACScan cytometer (BD), and results were quantified using FlowJo Software (Treestar). Cells transfected with the LoxP-PYMT lentivirus were lysed and analyzed by western blot using standard Odyssey system protocols. Primary antibodies against HA (mouse, 1:500 dilution, Santa Cruz 12CA5) were used to detect the HA-tagged PYMT, and primary antibodies against actin were used as a loading control (rabbit, 1:1000 dilution, Abcam ab1801). Anti-rabbit IRDye800 and anti-mouse IRDye680 secondary antibodies were used (1:10,000 dilution, Li-Cor). The antibody staining was visualized using the Odyssey Infrared Imaging System (Li-Cor).

In vitro 3D MEC culture

MECs were isolated from the mammary glands of 8-12-week-old Cre-expressing, mTmG, or FVB/NJ mice and frozen for storage as described previously (29). The same cell isolation protocol was used for all experiments using MECs.

To infect cells for 3D culture, MECs were thawed at 37^{o}C and plated in monolayer at a concentration of 2.5 x 10^5 cells per well in a 6-well plate, in MEC media (29). Thawed cells were allowed to adhere to the plate and recover overnight. Then, MECs were infected with high tighter lentivirus with a multiplicity of infection of 30, overnight. After infection, cells were washed five times with MEC media and trypsinized (0.05% trypsin/EDTA, Gibco). Single cells were transferred to a 24-well plate and were allowed to aggregate overnight in 1mL of MEC media. After aggregation, 500 MEC aggregates per 20μl Matrigel (BD Biosciences) were plated per well

in chamber slides (Millipore). Then, cysts were allowed to grow in epidermal growth factor (EGF) MEC media (5μg/mL insulin, 2.5nM mouse or human EGF, 100U/mL penicillin-streptomycin, DMEM-F12) for 5-7 days. Media was changed every other day. When the majority of aggregates formed hollow cysts, 4% paraformaldehyde in PBS was used to fix the cultures for 30 minutes at room temperature. Then cells were washed five times, for 5 minutes each with PBS, and permeabilized with 0.05% Triton-X 100 in PBS, overnight at 4^{o}C. Blocking was done overnight at 4^{o}C as well, using 3% BSA and 1% goat serum in PBS. Then, the cysts were stained with primary antibodies against K14 (1:400, rabbit, PRB-P-100, Covance) and K8 (1:50, rat, Troma-I, Developmental Studies Hybridoma Bank), overnight at 4^{o}C in block solution. Secondary antibodies Alexa Fluor 594 Chicken Anti-Rat IgG (1:1000, Invitrogen) and Alexa Fluor 488 Goat Anti-Rabbit IgG (1:1000, Invitrogen) were incubated with the cysts overnight at 4^{o}C in block solution. After washing the secondary antibodies off 5 times with PBS, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), chambers were removed from the slides, and the Matrigel cultures were cover-slipped. All immunofluorescent imaging was performed on an Olympus IX81 microscope using a Hamamatsu Photonics ORCA-ER camera. Fluorescent image recording and processing were performed using Slidebook 64 version 5.0.0.24.

MEC transplantation

To infect cells for transplantation experiments, MECs were thawed as described above and plated at a concentration of 2 x 10^{6} cells per well in a low adhesion 24-well plate, in MEC media. Cells were immediately infected with high titer lentivirus with a multiplicity of infection of 5, overnight. Following infection, cells were washed five times with MEC media and kept on ice until transplantation. For MECs infected with LoxP-Tomato lentivirus, 1 x 10^{5} cells were mixed with in 10μl of Matrigel (BD Biosciences) and injected into the fourth cleared inguinal mammary fat pads of 3-week-old FVB/NJ mice. For MMTV-Cre and FVB/NJ MECs infected with LoxP-PYMT lentivirus, 1 x 10^{5} cells were mixed with 10μl of Matrigel (BD Biosciences) and transplanted per mouse as described above. And for all MECs from mice that were bred with the mTmG Cre reporter, infected with LoxP-PYMT lentivirus, 2.5 x 10^{4} with 10μl of Matrigel (BD Biosciences),

and transplanted per mouse as described above. Tamoxifen was administered at two different time points to mice receiving transplants of CreER cells infected with the LoxP-PYMT letivirus. 5mg of tamoxifen (Sigma) dissolved in sesame oil (Sigma) was delivered through oral gavage, three times, once every other day. One cohort of transplants received tamoxifen at 1 week post transplantation (WK1), the second cohort was dosed at 4 weeks (WK4).

Mammary outgrowth and tumor analysis

LoxP-Tomato lentivirus infected MEC transplants were allowed to generate mammary outgrowths for 8 weeks. After 8 weeks, mammary glands were harvested, compressed between two glass slides, and imaged on a fluorescent dissecting scope. After imaging, tissues were dissociated as described previously [21], but were not trypsanized to single cells in order to preserve ductal structure for later staining and imaging. Next, organoids were embedded in Matrigel (BD Biosciences), fixed, stained, and imaged using the same methodology as the *in vitro* 3D MEC cyst culture experiments.

LoxP-PYMT lentivirus infected MEC transplants were allowed to grow into tumors until 2cm in diameter. Portions of the tumor tissues were flash frozen for later DNA and RNA extraction, fixed in 4% paraformaldehyde for later histology analysis, and dissociated to organoids and single cells for freezing, as described previously [21].

To amplify and sequence integrated lentiviral DNA in tumors generated with the LoxP-PyMT lentivirus, DNA was isolated from flash frozen tumor tissue using a Qiagen Dneasy Blood and Tissue Kit. PCR was performed on the isolated genomic DNA using the following primers: 5'-TATCCAGCACAGTGGCGG-3' (forward primer) and 5'-TTCTCCAGCAGTATGTGCG-3' (reverse primer). The PCR products were excised from an agarose gel using a Qiagen MinElute Gel Extraction Kit and Sanger sequenced using the forward primer.

References

1. Viale G. The current state of breast cancer classification. Ann Oncol 2012;23 Suppl 10:x207-10.

2. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98(19):10869-74.

3. Bertucci F, Birnbaum D. Reasons for breast cancer heterogeneity. J Biol 2008;7(2):6.

4. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12(5):R68.

5. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406(6797):747-52.

6. Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 2009;15(8):907-13.

7. Ince TA, Richardson AL, Bell GW, Saitoh M, Godar S, Karnoub AE, et al. Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 2007;12(2):160-70.

8. Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R, et al. BRCA1 Basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. Cell Stem Cell 2010;7(3):403-17.

9. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12(3):954-61.

10. Pittius CW, Hennighausen L, Lee E, Westphal H, Nicols E, Vitale J, et al. A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. Proc Natl Acad Sci U S A 1988;85(16):5874-8.

11. Wagner KU, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res 1997;25(21):4323-30.

12. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 2007;8(5):R76.

13. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nat Rev Cancer 2007;7(9):659-72.

14. Andrechek ER, White D, Muller WJ. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene 2005;24(5):932-7.

15. Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 2014;28(11):1143-58.

16. Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z. Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. Cell Stem Cell 2008;2(1):90-102.

17. Abremski K, Hoess R, Sternberg N. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. Cell 1983;32(4):1301-11.

18. Le Y, Miller JL, Sauer B. GFPcre fusion vectors with enhanced expression. Anal Biochem 1999;270(2):334-6.

19. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res 2001;10(6):545-53.

20. Kaanta AS, Virtanen C, Selfors LM, Brugge JS, Neel BG. Evidence for a multipotent mammary progenitor with pregnancy-specific activity. Breast Cancer Res 2013;15(4):R65.

21. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis 2007;45(9):593-605.

22. Deugnier MA, Moiseyeva EP, Thiery JP, Glukhova M. Myoepithelial cell differentiation in the developing mammary gland: progressive acquisition of smooth muscle phenotype. Dev Dyn 1995;204(2):107-17.

23. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science 2003;300(5617):329-32.

24. Xin HB, Deng KY, Rishniw M, Ji G, Kotlikoff MI. Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. Physiol Genomics 2002;10(3):211-5.

25. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature 2011;479(7372):189-93.

26. Fluck MM, Schaffhausen BS. Lessons in signaling and tumorigenesis from polyomavirus middle T antigen. Microbiol Mol Biol Rev 2009;73(3):542-63, Table of Contents.

27. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature 2012;490(7418):61-70.

28. Rubnitz J, Subramani S. The minimum amount of homology required for homologous recombination in mammalian cells. Mol Cell Biol 1984;4(11):2253-8.

29. Smith BA, Shelton DN, Kieffer C, Milash B, Usary J, Perou CM, et al. Targeting the PyMT Oncogene to Diverse Mammary Cell Populations Enhances Tumor Heterogeneity and Generates Rare Breast Cancer Subtypes. Genes Cancer 2012;3(9-10):550-63.

30. Marumoto T, Tashiro A, Friedmann-Morvinski D, Scadeng M, Soda Y, Gage FH, et al. Development of a novel mouse glioma model using lentiviral vectors. Nat Med 2009;15(1):110-6.

31. Friedmann-Morvinski D, Bushong EA, Ke E, Soda Y, Marumoto T, Singer O, et al. Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice. Science 2012;338(6110):1080-4.

32. Proia TA, Keller PJ, Gupta PB, Klebba I, Jones AD, Sedic M, et al. Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate. Cell Stem Cell 2011;8(2):149-63.

33. Guy CT, Cardiff RD, Muller WJ. Activated neu induces rapid tumor progression. J Biol Chem 1996;271(13):7673-8.

34. Krause S, Brock A, Ingber DE. Intraductal injection for localized drug delivery to the mouse mammary gland. J Vis Exp 2013(80).

35. Zhang L, Zhang B, Han SJ, Shore AN, Rosen JM, Demayo FJ, et al. Targeting CreER(T2) expression to keratin 8-expressing murine simple epithelia using bacterial artificial chromosome transgenesis. Transgenic Res 2012;21(5):1117-23.

Figure 3.1. Plasmid characterization and description of model. A. LoxP-Tomato lentiviral construct and Cre-mediated recombination. B. LoxP-PYMT lentiviral construct and Cre-mediated recombination. C. The LoxP-Tomato lentiviral construct expresses Tomato only after Cre-mediated recombination. Cells were transfected with LoxP-Tomato plasmid alone (left) or with the addition of a Cre-expression plasmid (right) and analyzed by FACS. D. The LoxP-PYMT lentiviral construct expresses PYMT only after Cre-mediated recombination. Cells were transfected with LoxP-PYMT plasmid alone or with the addition of a Cre-expression plasmid and analyzed by western blot. E. Model outline of targeting gene expression to specific MEC populations. Cells isolated from mice expressing Cre under the control of MEC population-specific promoters were infected with lentivirus *ex vivo*. Transduced cells were then transplanted in syngeneic background mice or grown in three-dimensional culture for analysis.

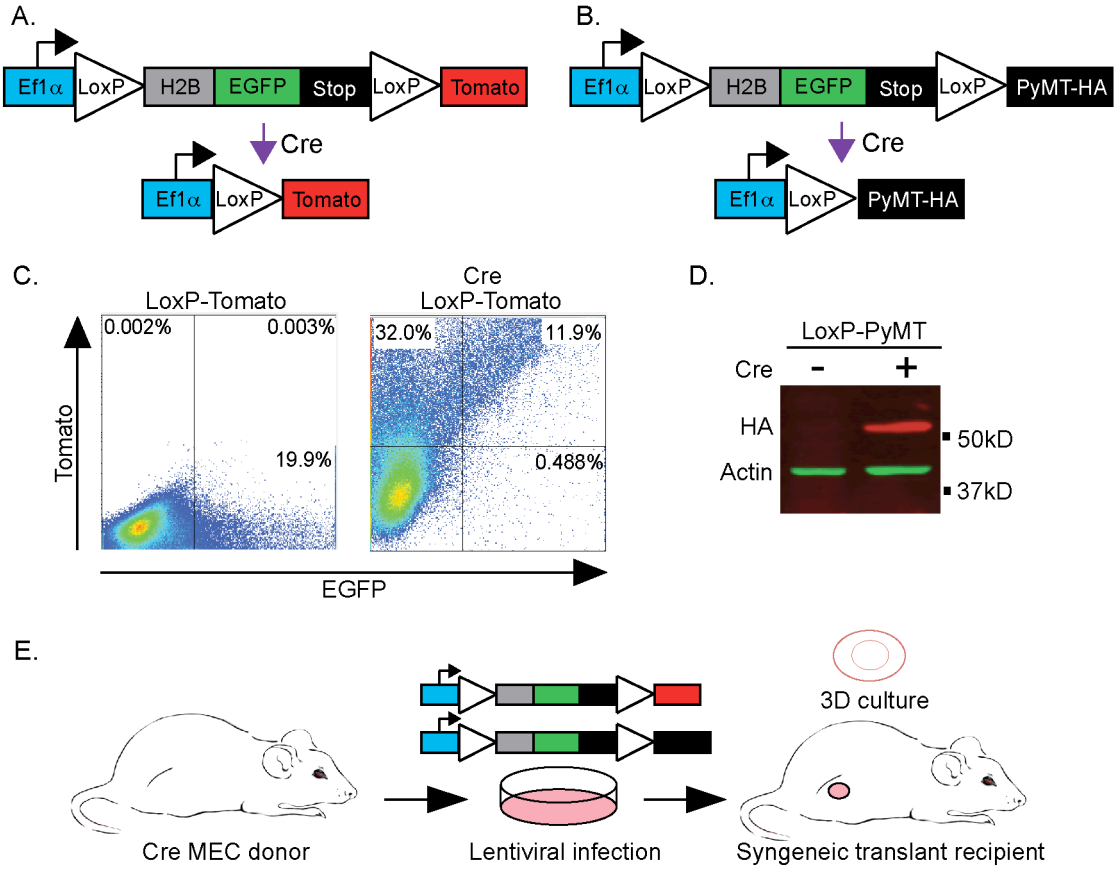

A.
Ef1α
LoxP
H2B
EGFP
Stop
LoxP
Tomato
Cre
Ef1α
LoxP
Tomato
B.
Ef1α
LoxP
H2B
EGFP
Stop
LoxP
PyMT-HA
Cre
Ef1α
LoxP
PyMT-HA
C.
LoxP-Tomato
Cre
LoxP-Tomato
0.002%
0.003%
19.9%
32.0%
11.9%
0.488%
Tomato
EGFP
D.
LoxP-PyMT
Cre
-
+
HA
Actin
50kD
37kD
E.
3D culture
Cre MEC donor
Lentiviral infection
Syngeneic translant recipient

Figure 3.2. MMTV-Cre MECs express Cre in the luminal and not basal cell lineage. A. *In vitro* MMTV-Cre;mTmG MEC organoid. GFP expression (green) indicates Cre activity, which overlaps with K8-positive luminal cells (red). B. *In vitro* MMTV-Cre;mTmG MEC organoid. GFP expression (green) indicates Cre activity, which does not overlap with K14-positive basal cells (red). C. MMTV-Cre;mTmG mouse mammary duct isolates. GFP expression (green) indicates Cre activity, which overlaps with K8-positive luminal cells (red). D. MMTV-Cre;mTmG mouse mammary duct isolates. GFP expression (green) indicates Cre activity, which does not overlap with K14-positive basal cells (red). Scale bars: 25μm.

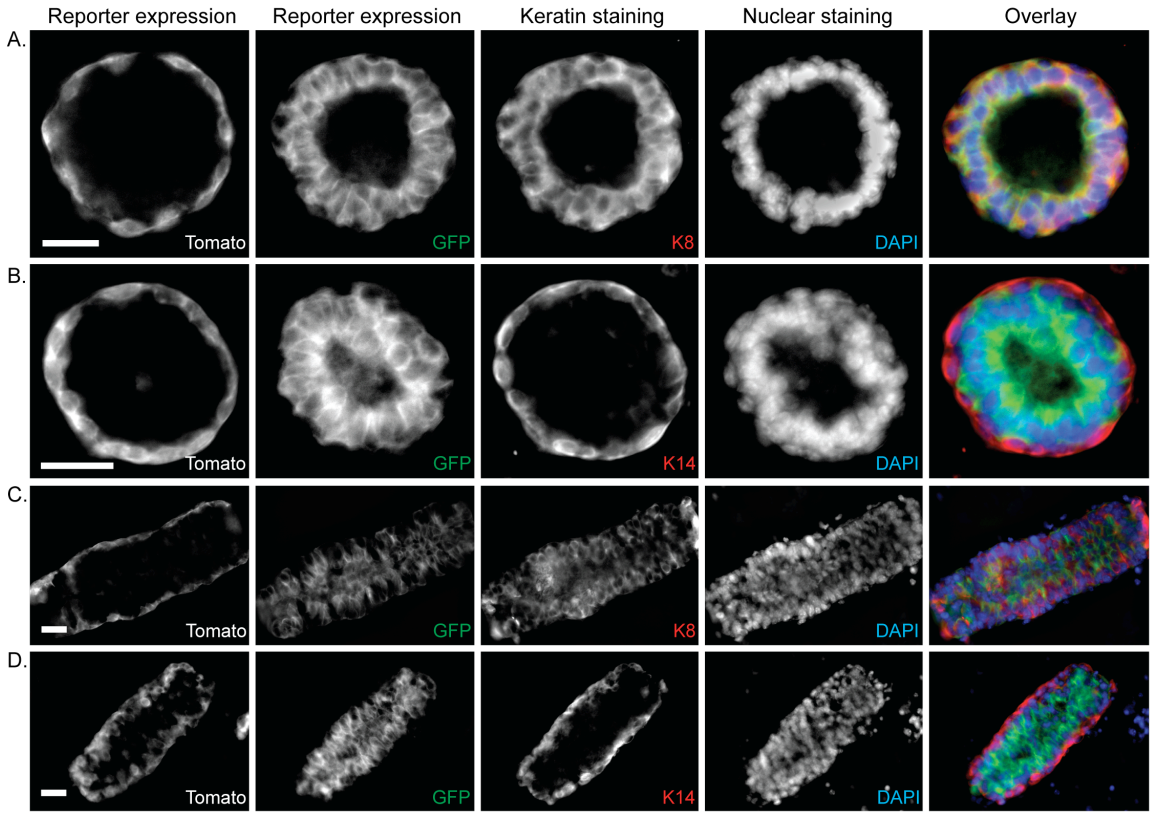

Reporter expression
Reporter expression
Keratin staining
Nuclear staining
Overlay
A.
B.
C.
D.
Tomato
GFP
K8
K14
DAPI

Figure 3.3. *In vitro* and *in vivo* luminal cell linage labeling using MMTV-Cre MECs infected with LoxP-Tomato lentivirus. A. Mammary cysts stained with luminal K8 marker (green). B. Mammary cysts stained with basal K14 (green) marker. C. Wholemount fluorescent imaging of a Tomato-positive mammary outgrowth. D. Summary of labeled outgrowths generated from MMTV-Cre MECs infected with LoxP-Tomato lentivirus. E. Mammary duct fragments stained with luminal K8 (green) F. Mammary duct fragments stained with basal K14 (green). Scale bars: 10μm.

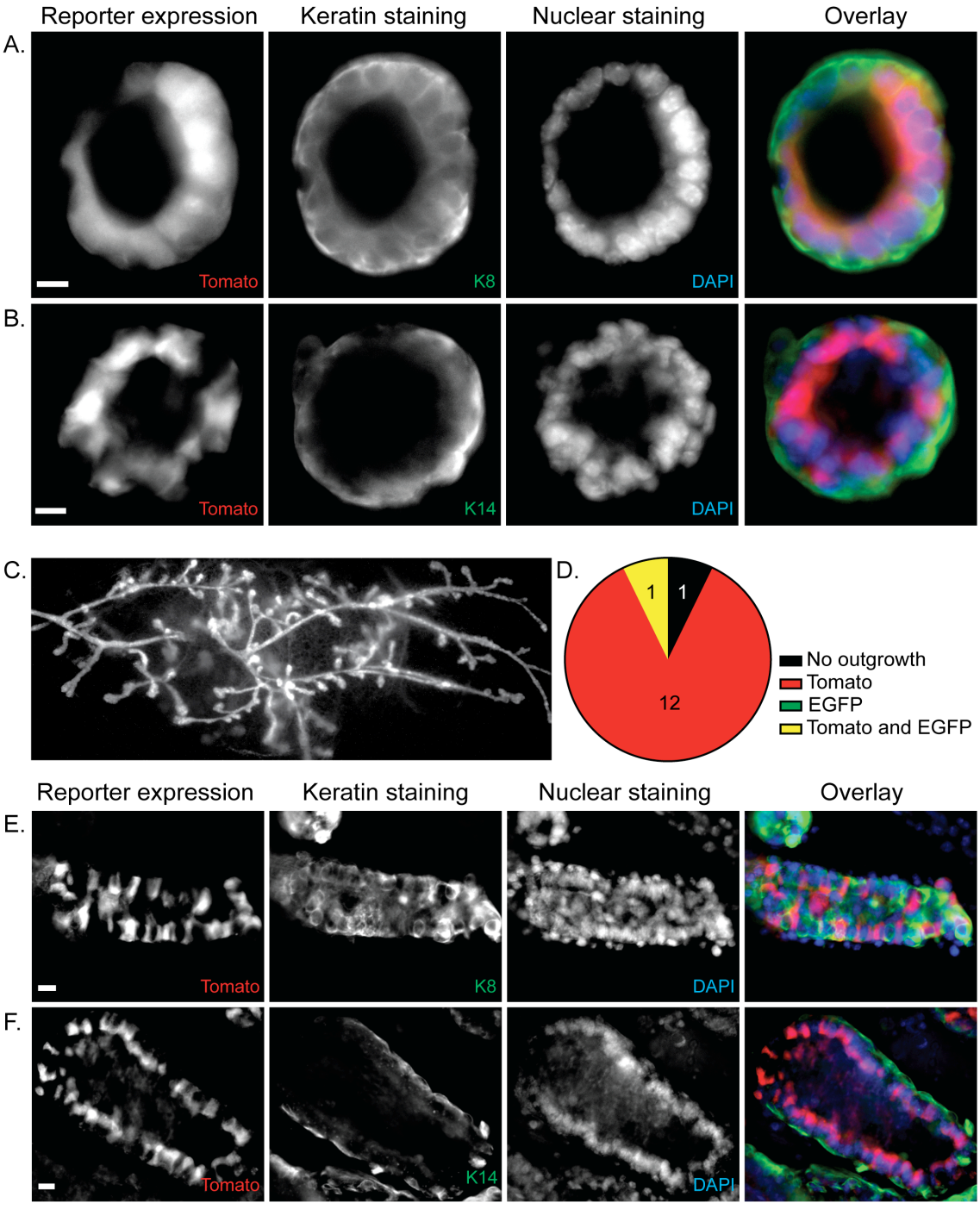

Reporter expression
Keratin staining
Nuclear staining
Overlay
A.
Tomato
K8
DAPI
B.
Tomato
K14
DAPI
C.
D.
1
1
12
No outgrowth
Tomato
EGFP
Tomato and EGFP
Reporter expression
Keratin staining
Nuclear staining
Overlay
E.
Tomato
K8
DAPI
F.
Tomato
K14
DAPI

Figure 3.4. *In vitro* lineage tracing using LoxP-Tomato lentivirus and MECs isolated from mice that express Cre under the control of basal and stem cell-specific promoters. MECs were cultured in 3D and stained with luminal K8 or basal K14 markers. A. K14-CreER MECs. B. SMMHC-Cre MECs. C. SM22α-Cre MECs. D. LGR5-CreER MECs. Scale bars: 10μm

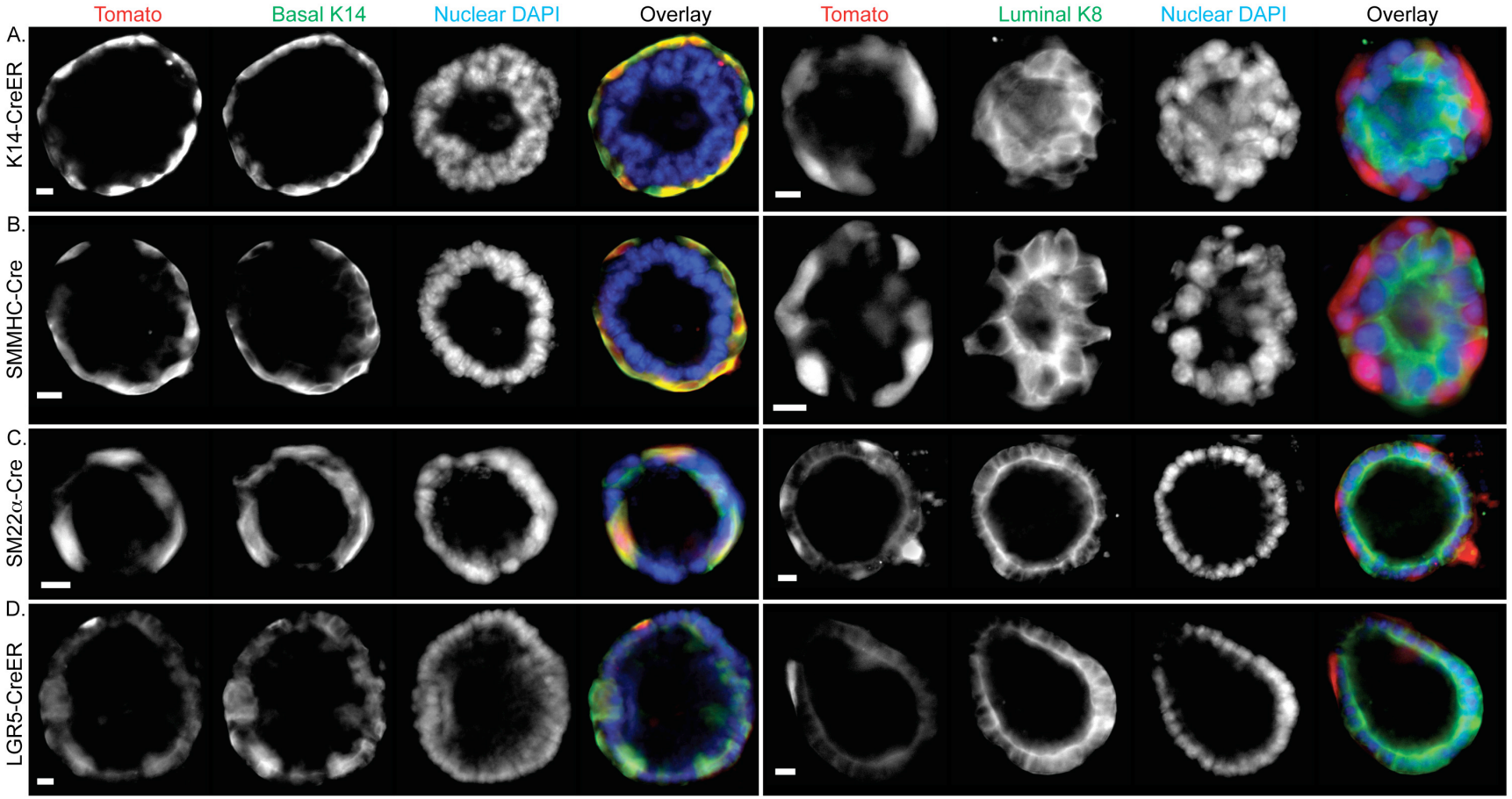
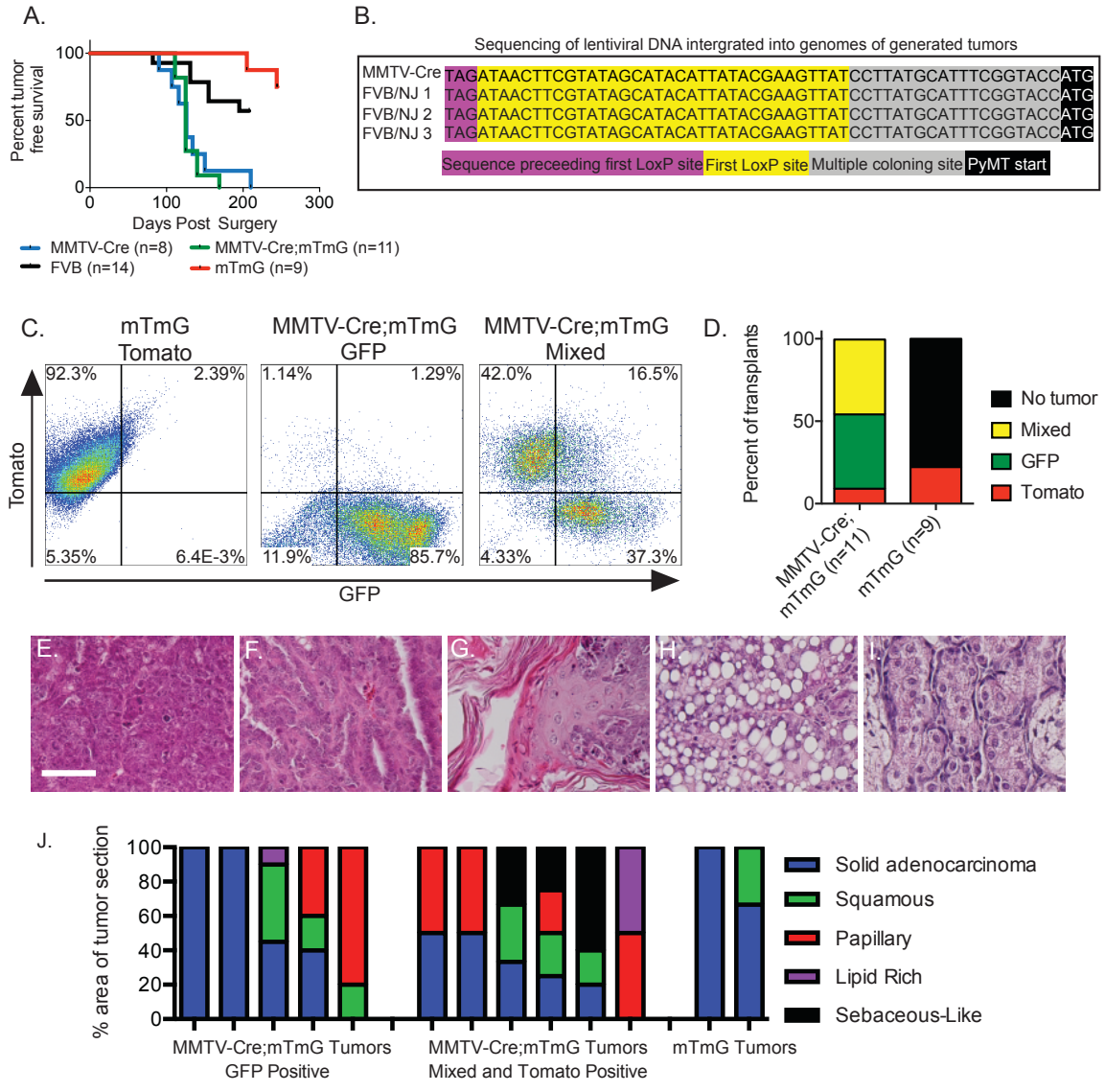
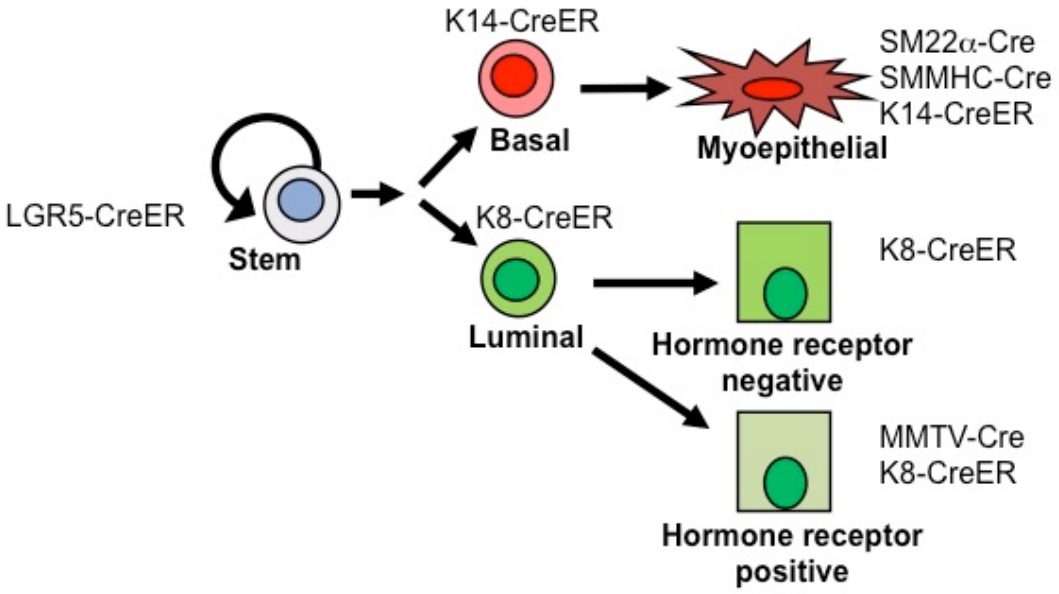

Tomato
Basal K14
Nuclear DAPI
Overlay
Tomato
Luminal K8
Nuclear DAPI
Overlay
A.
K14-CreER
B.
SMMHC-Cre
C.
SM22α-Cre
D.
LGR5-CreER

Figure 3.5. Generation of tumors from luminal cells using the LoxP-PYMT lentivirus. A. Tumor latencies of MMTV-Cre (blue), FVB/NJ (black), MMTV-Cre;mTmG (green), and mTmG (red) mouse MECs. B. Sequencing of integrated LoxP-PYMT virus from MMTV-Cre and FVB/NJ MEC tumors. C. FACS sorting demonstrating examples of Tomato-positive, GFP-positive, and mixed fluorescence tumors. D. Summary of tumor fluorescence from MMTV-Cre;mTmG and mTmG MECs. E-I. MMTV-Cre;mTmG and mTmG MEC tumor histology examples. Solid adenocarcinoma (E), papillary (F), squamous (G), lipid-rich (H), sebaceous-like (I). J. Summary of average histology area per tumor. Scale bar: 50μm


A.
Percent tumor free survival
100
50
0
0
100
200
300
Days Post Surgery
MMTV-Cre (n=8)
FVB (n=14)
MMTV-Cre;mTmG (n=11)
mTmG (n=9)
B.
Sequencing of lentiviral DNA intergrated into genomes of generated tumors
MMTV-Cre TAGATAACTTCGTATAGCATACATTATACGAAGTTATCCTTATGCATTTCGGTACCATG
FVB/NJ 1 TAGATAACTTCGTATAGCATACATTATACGAAGTTATCCTTATGCATTTCGGTACCATG
FVB/NJ 2 TAGATAACTTCGTATAGCATACATTATACGAAGTTATCCTTATGCATTTCGGTACCATG
FVB/NJ 3 TAGATAACTTCGTATAGCATACATTATACGAAGTTATCCTTATGCATTTCGGTACCATG
Sequence preceeding first LoxP site
First LoxP site
Multiple coloning site
PyMT start
C.
mTmG Tomato
MMTV-Cre;mTmG GFP
MMTV-Cre;mTmG Mixed
Tomato
GFP
92.3%
2.39%
5.35%
6.4E-3%
1.14%
1.29%
11.9%
85.7%
42.0%
16.5%
4.33%
37.3%
D.
Percent of transplants
100
50
0
MMTV-Cre; mTmG (n=11)
mTmG (n=9)
No tumor
Mixed
GFP
Tomato
E.
F.
G.
H.
I.
J.
% area of tumor section
100
80
60
40
20
0
MMTV-Cre;mTmG Tumors GFP Positive
MMTV-Cre;mTmG Tumors Mixed and Tomato Positive
mTmG Tumors
Solid adenocarcinoma
Squamous
Papillary
Lipid Rich
Sebaceous-Like

Supplementary Figure 3.1. Mammary epithelial cell populations and transgenic mice expressing Cre though cell lineage-specific promoters. Keratin 14 (K14) and leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) promoters are active in mammary gland basal and stem cells, respectively. The keratin 8 (K8) promoter is active in luminal progenitors and differentiated luminal cells (25,35). K14-CreER, LGR5-CreER, and K8-CreER transgenic mice express Cre that is regulated by a modified estrogen receptor (ER); and time-selective administration of tamoxifen can be used to activate Cre-recombinase activity in mammary stem cells, progenitors, or differentiated cells. Basal cells differentiate into myoepithelial cells express that smooth muscle 22-alpha (SM22α) smooth muscle myosin heavy chain (SMMHC) proteins; thus, SM22α-Cre and SMMHC-Cre should express Cre in differentiated basal myoepithelial cells (15,23,24). MMTV is a hormone-responsive promoter and induces Cre expression in hormone receptor-positive luminal cell lineages of MMTV-Cre transgenic mice (11,13).


K14-CreER
SM22α-Cre
SMMHC-Cre
K14-CreER
Basal
Myoepithelial
LGR5-CreER
Stem
K8-CreER
K8-CreER
Luminal
Hormone receptor
negative
MMTV-Cre
K8-CreER
Hormone receptor
positive

Supplementary Figure 3.2. FVB/NJ MEC outgrowths infected with the LoxP-Tomato lentivirus. A. Summary of outgrowth fluorescence. B. Outgrowth duct fragment stained with luminal K8 marker. C. Outgrowth duct fragment stained with basal 14 marker. Scale bars: 10μm.

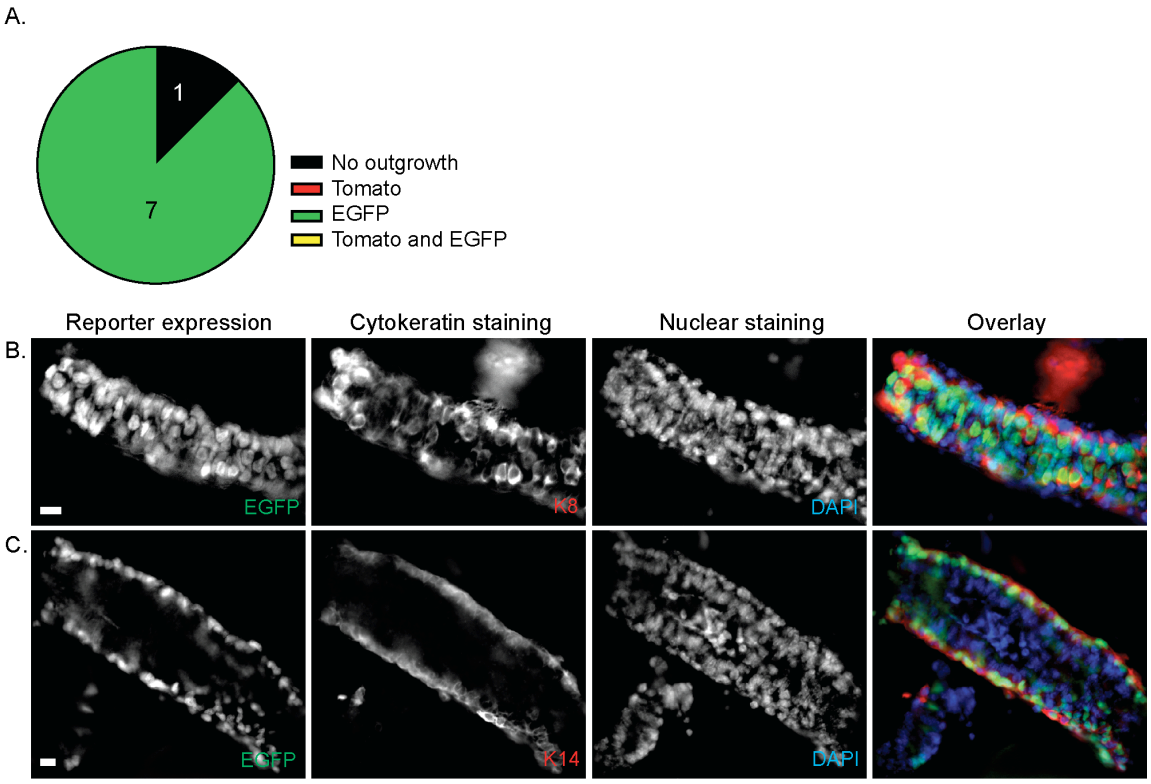

A.
1
7
No outgrowth
Tomato
EGFP
Tomato and EGFP
Reporter expression
Cytokeratin staining
Nuclear staining
Overlay
B.
EGFP
K8
DAPI
C.
EGFP
K14
DAPI

Table 3.1. Summary of latencies and fluorescence of tumors generated from Cre-expressing MECs

Cell of origin	Tamoxifen	Number of tumors	Average latency (days)
K14-CreER;mTmG	1 week	10 Tomato-positive	141.4
	4 week	5 Tomato-positive	163.4
	4 week	5 did not grow	
SM22α-Cre;mTmG		1 GFP-positive	113
		6 Tomato-positive	123.5
		1 mixed	140
		2 did not grow	
SMMHC-Cre;mTmG		8 GFP-positive	143.25
		1 Tomato-positive	162
		1 did not grow	
LGR5-CreER;mTmG	1 week	3 Tomato-positive	225
	1 week	7 did not grow	
	4 week	2 GFP-positive	171
	4 week	5 Tomato-positive	182.4
	4 week	2 did not grow	
K8-CreER;mTmG	1 week	3 GFP-positive	119
	1 week	3 Tomato-positive	137.5
	1 week	3 mixed	147
	1 week	2 did not grow	
	4 week	3 GFP-positive	72
	4 week	3 Tomato-positive	122
	4 week	4 mixed	80

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Breast cancer is a heterogeneous disease, which consists of several subtypes that have distinct pathologies and clinical outcomes (1-3). Several factors may lead to this disease diversity. First, various genetic alterations acquired during tumor progression may strongly influence tumor phenotype. In addition, intrinsic properties of the tumor cell of origin may also define tumor subtype. However, these factors may not be mutually exclusive.

Breast cancer development and progression have been extensively studied using mouse models. Several transgenic mouse mammary tumor models have demonstrated that different oncogenes expressed through the same promoter can give rise to distinct tumor types. For example, MMTV-PYMT mice give rise to a high proportion of molecularly luminal tumors, whereas MMTV-Wnt mice give rise to molecularly Basal tumors (2). These results indicate that oncogenes or mutations influence breast cancer subtype. However, most transgenic mouse mammary tumor models do not recapitulate the full heterogeneity observed in breast cancer (2,4) possibly due to the fact that most transgenic mice do not use promoters that can target oncogene expression to the broad spectrum of mouse mammary epithelial cell types. Consequently, transgenic mouse mammary tumor models are limited in their ability to test whether specific cell populations can contribute to distinct tumor phenotypes.

As an alternative to transgenic models, Smith et al. demonstrated that diverse mouse mammary tumors can be generated using lentiviral infection and mammary epithelial cell transplantation techniques (5). However, the question remained if specific mammary epithelial cell populations could give rise to unique tumor types. Therefore, to test how the tumor cell of origin influences breast cancer phenotype, this dissertation described several novel approaches

that utilize lentiviral infection and primary mammary epithelial cell transplantation to transform specific mammary epithelial cell populations.

First, we conducted a study to test how enriched stem, basal, luminal progenitor, and differentiated luminal, mammary epithelial cell (MEC) populations contribute to breast cancer phenotype. This was achieved by infecting mouse MECs with a previously described lentivirus containing the polyoma middle T antigen (PYMT) oncogene (5). Then, specific MEC populations were isolated by fluorescence activated cell sorting (FACS) and orthotopically transplanted to generate tumors. Our data show that all enriched populations produced tumors with equivalent latencies and gave rise to histologically diverse tumors. However, the prevalence of solid adenocarcinomas, squamous, papillary, and sebaceous-like histologies varied among the transformed populations. Also, molecularly diverse tumors resulted from most of the MEC populations, but luminal progenitors exclusively gave rise to tumors that resemble aggressive Basal-like breast cancers (2). Finally, compared to the other MEC populations, the differentiated luminal population produced tumors that were significantly less metastatic, possibly due to an inability of the tumor cells to escape the primary tumor site. These findings show that distinct mammary epithelial cells have the capacity to give rise to diverse tumor types; however, the tumor cell of origin influences breast cancer subtype and metastatic potential.

Typically, breast cancers with metaplastic histologies are much more aggressive than well-differentiated tumors that maintain some cellular organization similar to normal mammary tissue (6). Interestingly, we found that of the enriched populations, more differentiated luminal cells gave rise to a higher proportion of well-differentiated papillary and acinar tumors; which are characterized by their maintenance of basal and luminal cell organization throughout the tumor mass (6). Conversely, basal and stem cell-enriched populations gave rise to more metaplastic squamous tumors, consisting of disorganized epithelium intermixed with large keratinized regions (6). Of note, mammary stem cell maintenance relies heavily on Wnt signaling (7,8), and constitutive activation of β-catenin in mammary epithelial cells promotes squamous tumor growth (7,9,10). These findings suggest that stem cell properties may promote squamous tumor development. To test this hypothesis, PYMT and stem cell-specific transcription factors can be

overexpressed in luminal cells to generate tumors. Then tumor histology can be assessed to determine whether the additional expression of stem cell factors enhances squamous tumor development.

Estrogen receptor (ER) status is another factor that influences breast cancer prognosis and course of treatment (11). However, it is not known whether specific breast cell populations develop into ER-dependent tumors. Our data demonstrate that all MEC populations can give rise to ER positive tumors; however, we detected the highest proportion of ER expression in tumors derived from differentiated luminal cells. Stem cells, on the other hand, produced the lowest percent of ER positive tumors. These findings correlate with the hormone receptor profiles of normal MEC populations: differentiated luminal cells express the highest levels of ER, and stem cells express the lowest (12). Our tumor data suggest that breast cancer hormone receptor status may depend on the intrinsic properties of the tumor cell of origin. Therefore, further experiments need to be performed to test whether differentiated luminal cells are the tumor cells of origin for hormone-dependent breast cancers. Tumor dependence on estrogen signaling can be examined by treating tumor-bearing mice with the ER inhibitor tamoxifen (13), or by transplanting cells into ovariectomized mice (14).

Furthermore, some breast cancer subtypes are much more aggressive and metastatic than others (15). Our data demonstrate that tumors generated from differentiated CD133+ luminal cells are significantly less metastatic than other population-derived tumors. Therefore, the differentiation status of the tumor cell of origin may influence how aggressive and metastatic a breast cancer may be. Compounds that can activate mammary epithelial cell differentiation may promote the development of more benign tumor lesions from transformed cell populations. Because we have identified a population of mammary epithelial cells that gives rise to less aggressive tumors, our lentiviral infection model can be used to perform a chemical library screen to find drugs that can induce mixed mammary epithelial cells to differentiate into CD133+ luminal cells. More specifically, lentivirus-transduced mammary epithelial cells can be examined using flow cytometry to quantify the population types present after drug treatment. Then, compounds that promote an increased level of luminal CD133+ cell differentiation can be tested *in vivo* by

treating mice that have received transplants of mixed cells, transduced by the PYMT-expressing lentivirus. Following treatment, the tumor metastatic propensity and histology can be examined to determine if the identified drugs promote the development of more benign tumor lesions. Since we have shown that more differentiated cell populations give rise to less aggressive and well-differentiated ER+ tumors, this type of treatment may reduce the chance of a patient to relapse with an aggressive breast cancer type. Also, the identified compounds may be used in combination with standard of care chemotherapies to help maintain tumor cells in a more differentiated state, possibly resulting in decreased metastasis and better response to standard of care therapy.

We also found that tumor circulating cell levels were considerably lower in mice bearing differentiated luminal cell tumors, indicating that metastasis was decreased in these mice due to lack of primary site escape. Primary tumor site escape and metastasis can be promoted by the transition of tumor cells from an epithelial state to an invasive mesenchymal state (16). Basal and stem-enriched populations have been shown to express the highest levels of genes that regulate epithelial to mesenchymal transition (EMT), whereas differentiated luminal cells express high levels of genes active in differentiated mammary epithelial cells (17). Therefore, less differentiated MEC populations may be primed to undergo EMT and metastasize more readily. Differentiated luminal cells, on the other hand, may give rise to tumors that have more epithelial properties. As a result, these tumors may be less likely to undergo EMT and escape the primary tumor site. Analysis of cell morphology and EMT marker expression in differentiated luminal cell tumors may help determine whether these tumors lack EMT properties. This analysis can be followed by overexpression of genes that promote EMT, to determine whether metastasis can be enhanced in nonmetastatic differentiated luminal cell tumors.

Although the transformation of enriched MEC populations demonstrated that the tumor cell of origin influences several aspects of breast cancer phenotype, tumors were generated from enriched and not pure populations. As a result, it is possible that some tumors may have arisen from multiple population types. This may explain why we observed extensive inter and intra tumoral heterogeneity in our molecular and histological analyses. While limiting dilution

transplants may lead to more homogenous tumors, we also developed an additional model of targeting gene expression to specific mammary epithelial populations. This was achieved by combining lentiviral infection and transplantation methodologies with Cre-LoxP recombination. This approach is more cell specific than flow cytometry enrichment techniques.

Briefly, to target gene expression to specific MEC populations without the need for cell enrichment, the lentivirus was designed to express a gene of interest downstream of a LoxP-flanked translational stop cassette. Then MECs isolated from mice that express Cre under the control of population-restricted promoters were infected to activate gene expression in a cell specific manner. Using this approach, we performed *in vitro* and *in vivo* studies to demonstrate that a fluorescent reporter could be targeted to luminal, basal, or stem MEC populations. In addition, we were able to generate tumors from distinct populations using the PYMT oncogene. However, we also found that tumors could arise in the absence of Cre due to inappropriate homologous recombination at lentiviral DNA LoxP sites. As a solution, we demonstrated that adding a Cre reporter allele into the Cre lines could identify tumors derived from Cre-expressing cells.

First, we generated tumors from the luminal cell population using MECs isolated from mice expressing Cre through the mouse mammary tumor virus long terminal repeat (MMTV) promoter (18). Many of these tumors displayed solid adenocarcinoma histology; however, papillary and squamous tumors were also detected. Similar to the MMTV-PYMT transgenic mouse tumor model (19), the MMTV-Cre mouse MECs appear to preferentially develop into solid adenocarcinomas. However, more tumors need to be analyzed to conclude whether this finding is significant.

Next, tumors were generated from stem cells using MECs isolated from mice that express Cre through the leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) promoter (20). Furthermore, basal cells were targeted for transformation after transducing cells isolated from mice that express Cre though keratin (K14), smooth muscle 22-alpha (SM22α), and smooth muscle myosin heavy chain (SMMHC) promoters (20-23). Since we used a Cre reporter to label tumors derived from Cre-expressing cells, we were able to distinguish between tumors

that arose from Cre-mediated recombination and inappropriately pre-recombined virus. Unfortunately, few Cre-expressing cells gave rise to tumors from K14-Cre, SMMHC-Cre, and LGR5-Cre cell transplants. Therefore, additional tumors need to be generated to fully characterize the contribution of basal and stem cell populations to breast cancer phenotype.

Unexpectedly, inappropriate tumors arose in 90-100% of K14-Cre, SMMHC-Cre, and LGR5-Cre cell transplants. However, cells isolated from mice that do not express Cre generated tumors only 20% of the time, with significantly longer latencies, suggesting that there is some sort of advantage for aberrant tumor growth in transplants where any cells express Cre. We hypothesize that Cre-expressing cells may be initially transformed; however, these cells may interact with and enhance the transformation or growth of neighboring cell populations that do not express Cre. The neighboring cells may be more proliferative and outcompete the initial tumor. A recent finding supports the hypothesis that tumors interact with normal cells during cancer progression. Melo et al. demonstrated that microRNAs in exosomes secreted from breast cancer cells can transform normal neighboring populations (24). Cellular interactions through exosomes may also promote inappropriate tumor growth in our tumor transplants. To test this hypothesis, exosomes shed from tumor cells could be analyzed for PYMT protein or RNA; and cultured with normal cells to determine if they have transformative properties. Also, it would be interesting to see if labeled normal MECs can give rise to tumors when transplanted in conjunction with differentially labeled tumor cells. Studies like these may provide novel insights into the mechanisms of breast cancer development and progression.

Besides PYMT, the lentiviral Cre-LoxP system can be used to target other oncogenes to the mammary epithelium. For example, expressing Neu, a rat homolog of the human epidermal growth factor receptor 2 (HER2) (25), in specific MEC populations may demonstrate that distinct cell types preferentially give rise to tumors that resemble HER2-positive breast cancer. So far, we have successfully generated mouse mammary tumors using a lentivirus that expresses Neu downstream of the LoxP-flanked stop cassette. Unfortunately, these experiments were performed before we discovered that aberrant tumors could arise in the absence of Cre. Therefore, additional studies need to be performed with the use of a Cre reporter system (26).

Alternatively, our lentiviral methodology can be used to express transposable elements instead of oncogenes downstream of the LoxP-flanked cassette. Sleeping beauty transposable elements have been extensively used to perform forward genetic screens for cancer gene discovery. These transposable elements activate tumor growth through insertional mutagenesis, either by promoting endogenous proto-oncogene overexpression or tumor suppressor inhibition (27). Because transposable elements do not provide a growth advantage on their own, it is unlikely that rare pre-recombined lentiviruses would lead to tumor growth. However, when overexpressed in specific MEC populations, transposable elements may provide insights into the types of proto-oncogenes or tumor suppressors that drive tumorigenesis in distinct breast cancer cells of origin.

As discussed above, our lentiviral infection and transplantation approaches have several advantages to traditional transgenic models. First, we can target gene expression to specific cell populations in the mammary epithelium. Second, lentiviral constructs can be easily manipulated to express a broad variety of oncogenes. Also, our oncogene expression is restricted to the mammary epithelium, in an immune competent mouse. Therefore, we were able to detect metastases in distant organs, and can examine how the immune system may influence tumor growth and progression. However, our system has several drawbacks as well. For example, lentiviral integration may impact tumor subtype due to insertional mutagenesis, and the number of lentiviral integrations into host genome cannot be regulated. Also, transplantation of enriched populations gave rise to diverse tumor types, and it is not clear whether that was due to the cell of origin or lack of population purity. Finally, tumors generated with the Cre-LoxP lentiviral system arose in the absence of Cre activity, demonstrating that our oncogene expression is not tightly regulated in the lentivirus.

Several alternative approaches can be used to avoid the disadvantages of the lentiviral infection and transplantation models to generate tumors from specific mammary epithelial cell populations. In one such approach, an internal ribosome entry site and PYMT oncogene could be knocked into the mTmG Cre reporter mouse line downstream of green fluorescent protein (GFP) (26). This way, the oncogene would be integrated in one locus of the mouse genome, and would

not impact any essential genes by insertional mutagenesis. Also, the PYMT oncogene would only be expressed in the presence of Cre recombinase. To activate PYMT expression in specific mammary epithelial cell populations, adenoviruses expressing Cre under the control of cell lineage-specific promoters could be used to infect the mammary epithelium through intraductal injection (28). Since adenoviruses do not integrate into the host genome (29), insertinal mutagenesis due to viral integration would not be an issue. Also, intraductal injection of the virus would limit oncogene expression to the mouse mammary epithelium, and would also eliminate the need for backbreeding mice because syngeneic transplantation would not be necessary. Finally, since tumors would be fluorescently labeled, metastases and cellular interactions with non-tumor cells could be easily identified.

In summary, we have developed two mouse breast cancer models that can be used to target oncogene expression to specific MEC populations. First, we utilized lentiviral and MEC transplantation methodologies, combined with flow cytometry population enrichment, to generate tumors from basal, stem, and luminal cell populations. Our results show that diverse tumors can arise from specific MEC populations; however, the tumor cell of origin also influences breast cancer subtype and metastatic potential. Second, we demonstrated that lentiviral infection and transplantation techniques can be combined with Cre-LoxP recombination to transform specific MECs. However, we also determined that a Cre reporter system is essential in order to identify tumors arising from Cre-expressing cell populations.

References

1. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12(5):R68.

2. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 2007;8(5):R76.

3. Viale G. The current state of breast cancer classification. Ann Oncol 2012;23 Suppl 10:x207-10.

4. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. Nat Rev Cancer 2007;7(9):659-72.

5. Smith BA, Shelton DN, Kieffer C, Milash B, Usary J, Perou CM, et al. Targeting the PyMT Oncogene to Diverse Mammary Cell Populations Enhances Tumor Heterogeneity and Generates Rare Breast Cancer Subtypes. Genes Cancer 2012;3(9-10):550-63.

6. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: how special are they? Mol Oncol 2010;4(3):192-208.

7. Moumen M, Chiche A, Decraene C, Petit V, Gandarillas A, Deugnier MA, et al. Myc is required for beta-catenin-mediated mammary stem cell amplification and tumorigenesis. Mol Cancer 2013;12(1):132.

8. Zeng YA, Nusse R. Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell 2010;6(6):568-77.

9. Miyoshi K, Shillingford JM, Le Provost F, Gounari F, Bronson R, von Boehmer H, et al. Activation of beta -catenin signaling in differentiated mammary secretory cells induces transdifferentiation into epidermis and squamous metaplasias. Proc Natl Acad Sci U S A 2002;99(1):219-24.

10. Teuliere J, Faraldo MM, Deugnier MA, Shtutman M, Ben-Ze'ev A, Thiery JP, et al. Targeted activation of beta-catenin signaling in basal mammary epithelial cells affects mammary development and leads to hyperplasia. Development 2005;132(2):267-77.

11. Lapidus RG, Nass SJ, Davidson NE. The loss of estrogen and progesterone receptor gene expression in human breast cancer. J Mammary Gland Biol Neoplasia 1998;3(1):85-94.

12. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. J Cell Biol 2007;176(1):19-26.

13. Jordan VC. Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br J Pharmacol 1993;110(2):507-17.

14. Idris AI. Ovariectomy/orchidectomy in rodents. Methods Mol Biol 2012;816:545-51.

15. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98(19):10869-74.

16. Nieto MA. The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu Rev Cell Dev Biol 2011;27:347-76.

17. Lim E, Wu D, Pal B, Bouras T, Asselin-Labat ML, Vaillant F, et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. Breast Cancer Res 2010;12(2):R21.

18. Andrechek ER, White D, Muller WJ. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene 2005;24(5):932-7.

19. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12(3):954-61.

20. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature 2011;479(7372):189-93.

21. Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. Science 2003;300(5617):329-32.

22. Xin HB, Deng KY, Rishniw M, Ji G, Kotlikoff MI. Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. Physiol Genomics 2002;10(3):211-5.

23. Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 2014;28(11):1143-58.

24. Melo SA, Sugimoto H, O'Connell JT, Kato N, Villanueva A, Vidal A, et al. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. Cancer Cell 2014;26(5):707-21.

25. Guy CT, Cardiff RD, Muller WJ. Activated neu induces rapid tumor progression. J Biol Chem 1996;271(13):7673-8.

26. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis 2007;45(9):593-605.

27. Mann MB, Jenkins NA, Copeland NG, Mann KM. Sleeping Beauty mutagenesis: exploiting forward genetic screens for cancer gene discovery. Curr Opin Genet Dev 2014;24:16-22.

28. Tao L, van Bragt MP, Laudadio E, Li Z. Lineage tracing of mammary epithelial cells using cell-type-specific cre-expressing adenoviruses. Stem Cell Reports 2014;2(6):770-9.

29. Anderson RD, Haskell RE, Xia H, Roessler BJ, Davidson BL. A simple method for the rapid generation of recombinant adenovirus vectors. Gene Ther 2000;7(12):1034-8.